



Mechanisms involved in the blood–testis barrier increased permeability induced by EMP

Xiao-Wu Wang^a, Gui-Rong Ding^{a,*}, Chang-Hong Shi^b, Li-Hua Zeng^a, Jun-Ye Liu^a, Jing Li^a, Tao Zhao^a, Yong-Bin Chen^a, Guo-Zhen Guo^{a,*}

^a Department of Radiation Medicine, Faculty of Preventive Medicine, The Fourth Military Medical University, Xi'an 710032, China

^b Animal Center, The Fourth Military Medical University, Xi'an 710032, China

ARTICLE INFO

Article history:

Received 14 May 2010

Received in revised form 1 July 2010

Accepted 3 July 2010

Available online 13 July 2010

Keywords:

Electromagnetic pulse

Blood–testis barrier

ZO-1

Occludin

TGF- β 3

Antisperm antibody

ABSTRACT

The blood–testis barrier (BTB) plays an important role in male reproductive system. Lots of environmental stimulations can increase the permeability of BTB and then result in antisperm antibody (AsAb) generation, which is a key step in male immune infertility. Here we reported the results of male mice exposed to electromagnetic pulse (EMP) by measuring the expression of tight-junction-associated proteins (ZO-1 and Occludin), vimentin microfilaments, and transforming growth factor-beta (TGF- β 3) as well as AsAb level in serum. Male BALB/c mice were sham exposed or exposed to EMP at two different intensities (200 kV/m and 400 kV/m) for 200 pulses. The testes were collected at different time points after EMP exposure. Immunofluorescence histocytochemistry, western blotting, laser confocal microscopy and RT-PCR were used in this study. Compared with sham group, the expression of ZO-1 and TGF- β 3 significantly decreased accompanied with unevenly stained vimentin microfilaments and increased serum AsAb levels in EMP-exposed mice. These results suggest a potential BTB injury and immune infertility in male mice exposed to a certain intensity of EMP.

© 2010 Elsevier Ireland Ltd. All rights reserved.

1. Introduction

Exposure to electromagnetic fields such as electromagnetic pulse (EMP) including household appliances is becoming an inevitable environmental stress and a public concern on health. The male reproductive system is one of the most sensitive targets that may be affected by exposure to electromagnetic fields (Nisarg et al., 2009). The data from an epidemiological study showed that male infertility were associated with increasing self-reported exposure to radiofrequency electromagnetic fields (Baste et al., 2008). It has been reported that immunological factors are involved in human infertility and 20–30% of male infertility is associated with the generation of antisperm antibodies (AsAb) (Dimitrova et al., 2002). AsAb generated in men and/or women is able to decrease male fertility by inhibiting sperm functions required for

fertilization (Bohring and Krause, 2005). The blood–testis barrier (BTB) plays an important physiological role in male reproductive system. An increase in BTB permeability is known to enhance AsAb production and then result in infertility in males. A human population-based prospective cohort study showed that exposure to some kinds of electromagnetic fields could increase BTB permeability (Li et al., 2002; Slama et al., 2004). In addition, it was also reported that Evans Blue (EB) and lanthanum nitrate may permeate the BTB and increase its permeability following exposure to EMP at certain intensity and high-power microwave exposure (Li et al., 2002; Wang et al., 2008). EMP is a short high-voltage pulse with an extremely fast rising time and a broad bandwidth. EMP signals can be generate by strong electrical field apparatus such as high pressure gas switch and Tesla transformer generators in some occupational conditions. About the exposure limit of this kind of signal, there is big difference in different countries or associations. For example, in occupational case, the IEEE (Institute of Electrical and Electronics Engineers) limit exposure for whole body (including heads) is 100 kV/m (IEEE-SA Standards Board, 1999), however, the ICNIRP (International Commission on Non-Ionizing Radiation Protection) reference level for occupational exposure to time vary electric field is 137 kV/m (ICNIRP, 1998). To explore the mechanisms of EMP-induced BTB permeability change and its influence on male reproductivity, we observed the effects of EMP on tight-junction-associated proteins (ZO-1, Occludin),

Abbreviations: BTB, blood–testis barrier; AsAb, antisperm antibody; EMP, electromagnetic pulse; TGF- β , transforming growth factor-beta; ZO, zonula occludens proteins; CTEM, Gigahertz Transverse Electromagnetic; PAGE, polyacrylamide gel electrophoresis; ECL, electrochemiluminescence; DMEM, Dulbecco's modified Eagle medium; RT-PCR, reverse transcription polymerase chain reaction; EB, evans Blue; TJ, tight junction; MDCK, Madin–Darby canine kidney; BBB, blood–brain barrier.

* Corresponding authors. Tel.: +86 29 84774873; fax: +86 29 84774873.

E-mail addresses: dingzhao@fmmu.edu.cn (G.-R. Ding), guozhen@fmmu.edu.cn (G.-Z. Guo).

vimentin microfilaments, and transforming growth factor-beta (TGF- β 3), which are associated with the BTB permeability. The level of AsAb in mice exposed to EMP was also examined in this study.

2. Materials and methods

2.1. EMP exposure

The EMP (peak-intensity 200 kV/m and 400 kV/m, rise-time 10 ns, pulse-width 350 ns) was generated by a spark gap pulse generator and transmitted into a Gigahertz Transverse Electromagnetic (GTEM) cell. The average specific absorption rate (SAR) used for the animal was 0.04 mW/kg, which was determined according to the Radiofrequency Radiation Dosimetry Handbook (4th ed.). The EMP generator and the GTEM cell were both devised by the Department of Mechanical Engineering, Southeast University (Nanjing City, Jiangsu Province, China) (Chen et al., 2010). Each mouse was given an equilibration period of 5 min before EMP exposure in GTEM cell. Sham exposure was conducted in the same GTEM cell without the generation of EMP. The animals were sham or exposed to EMP for 200 pulses with 0.5 Hz repetitive rate. The temperature in GTEM cell was kept $26 \pm 0.5^\circ\text{C}$. The rectal temperature of animals was $38.42 \pm 0.33^\circ\text{C}$ and $38.56 \pm 0.38^\circ\text{C}$ before and after EMP exposure, respectively.

2.2. Animals

Male BALB/c mice (7–8 weeks old, weighing 21 ± 2 g) were purchased from the Experimental Animal Center, Fourth Military Medical University. All studies were performed with the approval of the experimental animal care committee of the Fourth Military Medical University. The animals were maintained in standard laboratory conditions, acclimatized for 1 week, and then used for experimentation.

2.3. Experimental groups

For examining AsAb level in the serum after EMP exposure, 360 mice were divided randomly into four groups as follows: sham group, positive control group 200 kV/m group and 400 kV/m group, 90 animals in each group. For determining the expression level of ZO-1, Occludin, vimentin microfilaments and transforming growth factor-beta3 (TGF- β 3) after 200 kV/m EMP exposure, 24 mice were divided randomly into four groups ($n=6$) as follows: one sham group and three different time points groups (1 h, 2 h, and 6 h after EMP exposure).

2.4. AsAb induction and detection

AsAb were induced in positive group as previously described with a modification (Stern et al., 1994). Briefly, the mice were immunized by subcutaneously injecting of 0.5 ml sperm mixture and booster injections were administered 15 days later. Mice in other groups were sham exposed or exposed to EMP at two different intensities (200 kV/m or 400 kV/m) for 200 pulses. Peripheral blood were withdrawn from the eyes at 15 days after EMP exposure, then serum was collected and stored at -20°C . The serum AsAb level was detected using a commercial mouse antisperm antibody ELISA kit (Usclnife Company, Wuhan, China) according to the manufacturer's instructions.

2.5. Immunofluorescence staining

At 1 h, 2 h and 6 h after EMP exposure, six animals from sham or each time point group were killed. The testes were quickly dissected and stored at -80°C . Thereafter, testes were embedded in OCT cryostat-embedding compound (Tissue-Tek, Torrance, CA) and 10- μm -thick coronal sections were cut, then fixed in cold acetone and stored at -20°C . Endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide for 15 min and non-specific binding was blocked by incubating with 10% goat serum for 30 min. The sections were then incubated overnight at 4°C with primary antibodies (rabbit anti-ZO-1 polyclonal antibody 1:40 was obtained from Zymed Lab Inc., USA) followed by incubation with FITC-labeled secondary antibodies for 1 h (goat anti-rabbit IgG 1:200 was purchased from Sino-American Biotechnology Company, China). Immunofluorescence images were observed under a fluorescence microscope.

2.6. Western blot analysis

Approximately 200 mg of the tissue was lysed in a buffer containing 1% NP-40, 0.2 mol/L Tris-HCl (pH 8.0), 0.15 mol/L NaCl, 0.1% $\text{Na}_2\text{S}_2\text{O}_8$, and a protease inhibitor cocktail (0.017 mg/mL aprotinin, 1.0 mmol/L PMSF, 1.0 mmol/L Na_3VO_4 , and 0.1 mmol/L CaCl_2) and protein was extracted. Aliquots of the lysates were stored at -80°C for further experiments. The protein concentrations were determined by a Coomassie blue dye-binding assay (Bio-Rad, Company, USA). For this, an equal volume of $2\times$ SDS loading buffer was added, and the samples were boiled for 3 min. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out in a vertical slab gel apparatus (Bio-Rad). 20 μl protein was separated by SDS-PAGE at 80 V for 1.5 h and transferred to nitrocellulose filters. The filters were blocked with TBST

buffer (10 mM Tris-HCl at pH 8.0, 0.15 M NaCl, and 0.05% Tween 20) containing 5% skimmed milk and incubated with primary antibody to ZO-1, Occludin (1:150) (rabbit anti-Occludin polyclonal antibody from Zymed Lab Inc., USA), or vimentin (1:200) (monoclonal anti-vimentin antibody from Sigma, USA) at room temperature for 1 h followed by the addition of anti-rabbit IgG and anti-mouse IgG (Amersham-Pharmacia Biotech). The blots were visualized with electrochemiluminescence (ECL) kit (PIERCE, Biotechnology, Inc.).

2.7. Confocal microscopy and image analysis of Sertoli cells

Primary Sertoli cells were isolated from 15-day-old BALB/c mice (Cheng et al., 1986; Grima et al., 1997) and cultured at a cell density of 0.5×10^6 cells/cm² on Matrigel-coated 12-well dishes in serum-free Ham F12 nutrient mixture (F12) and Dulbecco's modified Eagle medium (DMEM) (1:1, v/v), as previously described (Lui et al., 2001). Cells were then incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 (v/v). Purified cells (>95%) were hypotonically treated with 20 mM Tris at pH 7.4 for 2.5 min to lyse contaminating germ cells for 36 h (Lipshultz et al., 1982). Thereafter, the Sertoli cell epithelium was washed twice to remove cellular debris. The media were replaced every 24 h and the cells were incubated for additional 6 or 7 days. The cultures were terminated at the specified time points for Sertoli cell identification. The identified Sertoli cells were seeded on coverslips and exposed to EMP (200 kV/m, 200 pulses). At 1 h, 2 h, and 6 h after EMP exposure, the cells were fixed in 4% paraformaldehyde, followed by double staining for vimentin and nuclei, and finally were examined under Olympus Fluoview BX61 confocal microscope.

2.8. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated using Trizol reagent (Invitrogen) from 100 mg testicular tissue from each group according to the manufacturer's instructions, dissolved in diethyl pyrocarbonate-treated water, and quantitated using a spectrophotometer. The purity of the RNA was assessed by spectrophotometry ($A_{260}/A_{280} > 1.8$). Reverse transcription (RT) of the RNA followed by polymerase chain reaction (PCR) was performed to detect TGF- β 3 gene expression (Cantz et al., 2003). cDNA was synthesized from the total RNA samples using an SYBR Green I Real Time PCR Kit (Bioer Technology Co., Ltd., Hangzhou, China). A quantity of 50 μL of PCR reaction mixture contained 4 μL of the RT product as the template, 25 μL $2\times$ PCR buffer, 0.3 μL (3U) Tap DNA polymerase, and 0.5 μL (10 pM) primers. The primers used were synthesized by AuGCT Biotechnology Company (Beijing, China), and their sequences were as follows: TGF- β 3 primers, sense 5'-GCTCTCCAGATACCTGAC-3' and antisense 5'-GTTGGACCTCTCTTGACGA-3' (440 bp) (Konrad et al., 2006). The PCR protocol was as follows: 95°C for 2 min for pre-denaturing; followed by 40 cycles at 94°C for 20 s, 55°C for 20 s, 72°C for 30 s; and a final extension at 72°C for 10 min. Fluorescence measurements were obtained for every cycle. β -Actin was used as the internal control.

2.9. Statistical analysis

The AsAb data was analysed by χ^2 test, other data were examined by ANOVA followed by hoc pairwise comparisons and Dunnett's *t*-test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. AsAb level in the serum

As shown in Fig. 1, AsAb were not detected in serum of sham-exposed mice, about 90% mice in positive control group (sperm-immunized group) showed AsAb positive in serum. After EMP exposure, there were 6.7% and 12.2% mice showed AsAb positive in serum in 200 kV/m and 400 kV/m group respectively, which were statistically different from those of the sham-exposed mice ($P < 0.05$ and 0.01 , respectively). Thus, our results showed that exposure to EMP could induce AsAb production in mice.

3.2. ZO-1 and Occludin expression level in EMP-treated mice

The immunofluorescence intensity of ZO-1 in sham, 1 h, 2 h, 6 h group were 17.23 ± 3.06 , 14.74 ± 2.65 , 11.92 ± 2.61 and 15.36 ± 1.87 , respectively. Compared with the sham, ZO-1 expression level decreased at 1 h, and reached the peak at 2 h ($P < 0.05$). Then ZO-1 level began to recover at 6 h after EMP exposure (Fig. 2), which was consistent with the results from western blot analysis

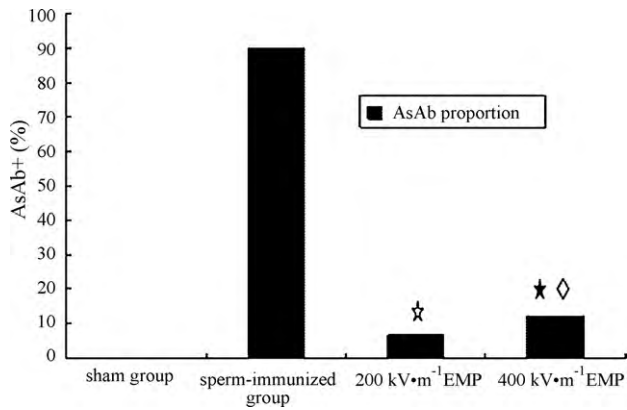


Fig. 1. Differences of AsAb production in mouse serum among different treatment groups. (☆) vs. sham group, $P < 0.05$; (*) vs. positive control group (sperm-immunized group), $P < 0.01$; (◇) vs. the 200 kV m⁻¹ exposed group, $P < 0.05$.

(Fig. 3). The expression level of Occludin did not change obviously at different time points after EMP exposure compared with sham (Fig. 4).

3.3. Morphological alterations of Sertoli cells

3.3.1. Phase contrast microscopy and HE staining

The cell body of Sertoli cells increased in size after 48 h of culturing, exhibiting a membrane-like covering on the wall of the culture flask. The nuclei were circular or oval and situated in the center or at a slight deviation. Numerous granules and vacuoles were observed in the cytoplasm. The number of cell processes increased and they were closely interconnected (Fig. 5a and b).

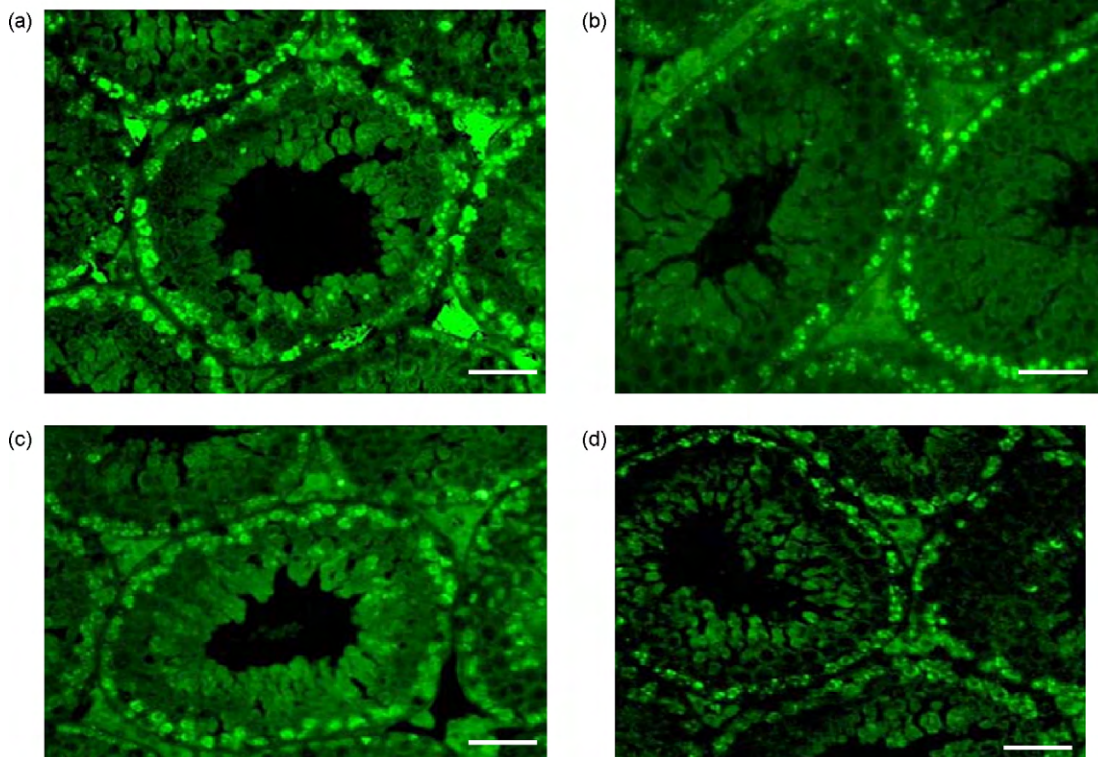


Fig. 2. Immunofluorescence results of ZO-1 in mice testicular tissues at different time points (1 h, 2 h and 6 h) after EMP exposure (200 kV/m, 200 pulses). The experiment was replicated at least twice (Bar = 40 μm).

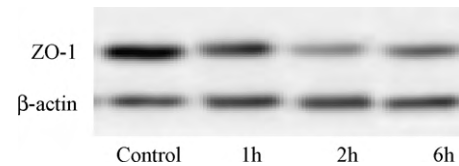


Fig. 3. Western blot results of ZO-1 in mice testicular tissues at different time points (1 h, 2 h and 6 h) after exposure to EMP (200 kV/m, 200 pulses). The experiment was replicated at least twice.

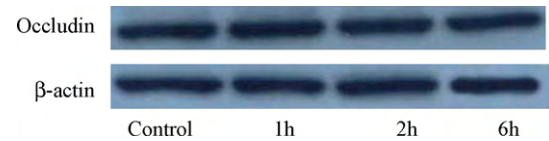


Fig. 4. Expression of Occludin in mice testes at different time points after exposure to EMP (200 kV/m, 200 pulses). The experiment was replicated at least twice.

3.3.2. Feulgen staining

The Sertoli cells cultured for 8 days were subjected to Feulgen staining. The results showed that the cytoplasm of Sertoli cell was not stained, and the nucleus was stained slightly. Stained granular particles were observed in the nucleus, which were identified as satellite nucleosomes around the nuclei (Fig. 5c).

3.3.3. Fas-L expression

Functional Sertoli cells naturally and stably express a high level of Fas-L (Jiang et al., 2002). Both fibroblasts and sperm cells did not express Fas-L. Therefore, Fas-L can be used as a marker for identifying Sertoli cells, in this study, we found that the cultured Sertoli cells were Fas-L immune-positive (Fig. 5d).

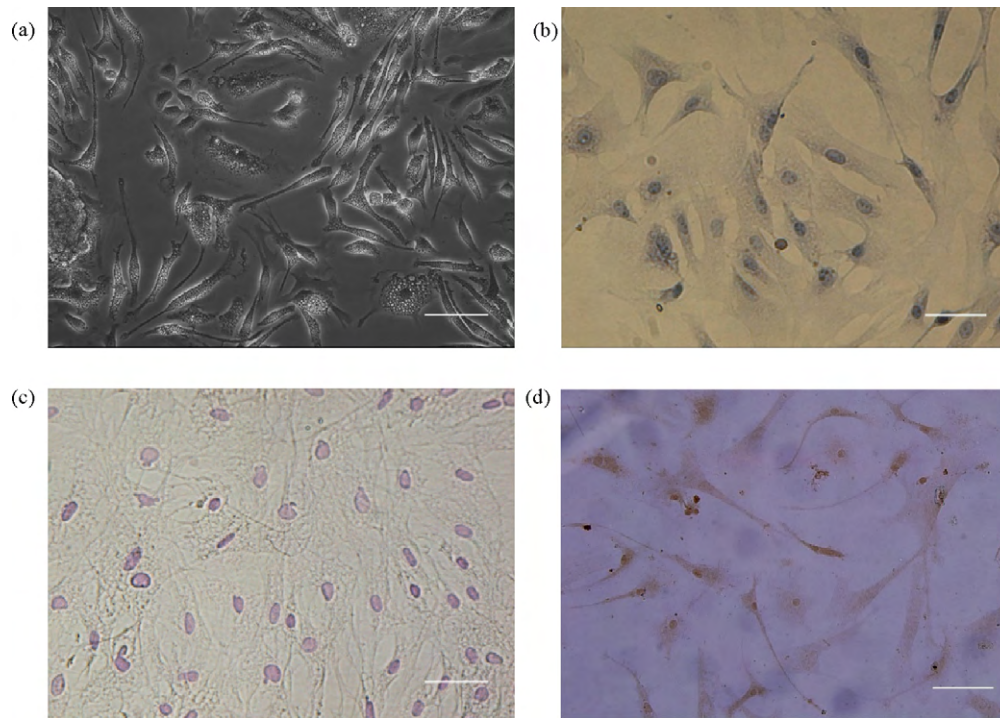


Fig. 5. Sertoli cells on day 7 of culture. (a) Pictures were taken under an inverted phase contrast microscope; (b) HE-stained Sertoli cells; (c) Feulgen-stained Sertoli cells; (d) Fas-L-stained Sertoli cells (Bar = 80 μ m).

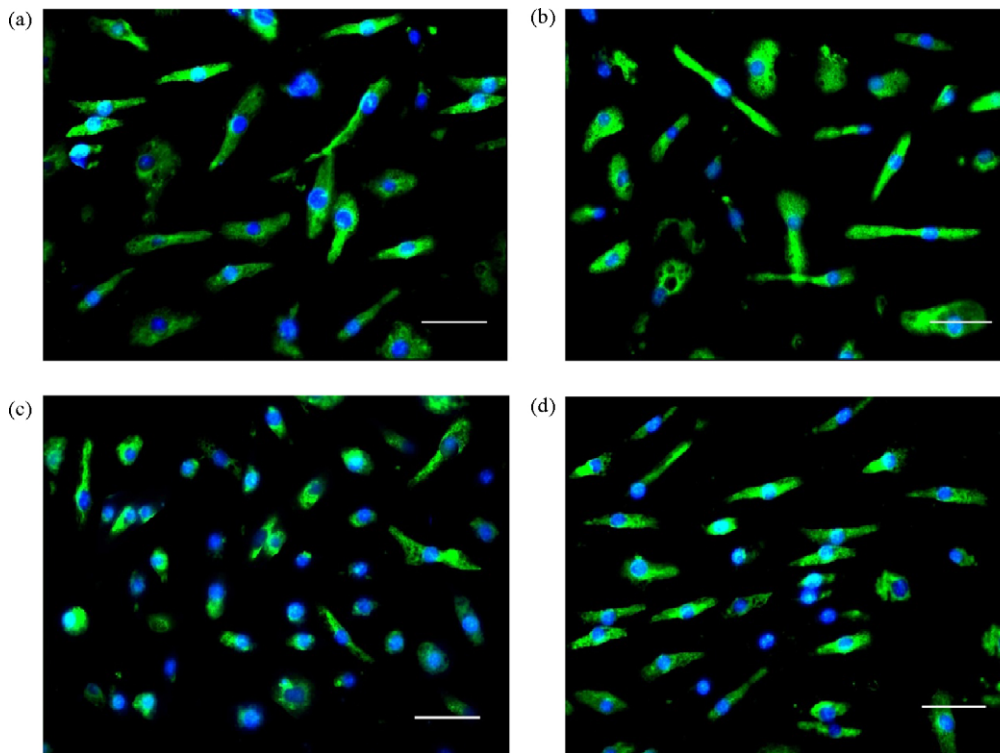


Fig. 6. Vimentin distribution in Sertoli cells. (a) Sham exposure group; (b) 1 h after EMP exposure (200 kV/m, 200 pulses); (c) 2 h after EMP exposure (200 kV/m, 200 pulses); (d) 6 h after EMP exposure (200 kV/m, 200 pulses). All the experiments were replicated at least twice (Bar = 80 μ m).

3.4. Vimentin expression in cultured Sertoli cells

The results showed that the Sertoli cells from the primary culture in sham group expressed vimentin at a higher level (Fig. 6a) and showed typical internal vimentin distribution. Vimentin extended radially from the perinuclear cytoplasm to the surrounding cell

membranes until it reached the cell membrane. Stained microfilaments of vimentin were consistent in length, form, and staining. In contrast, EMP exposure at 200 kV/m for 200 pulses resulted in changes in the distribution of vimentin in Sertoli cells. The cells exhibited a definite disorder and were not as consistent and regular as those in sham group. The length of the vimentin microfila-

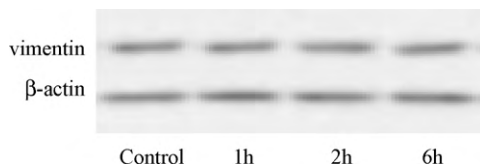


Fig. 7. Expression of vimentin protein in mice testicular tissues at different time points (1 h, 2 h and 6 h) after exposure to EMP (200 kV/m, 200 pulses). The experiment was replicated at least twice.

ments was decreased with an uneven staining at 1 h and 2 h after EMP exposure while at 6 h the microfilament length of vimentin increased with staining identical to that of the normal control group (Fig. 6). Western blot results showed the expression of vimentin had no obvious change after EMP exposure (Fig. 7).

3.5. TGF- β 3 mRNA expression

Compared with the sham, the TGF- β 3 mRNA level in testicular tissue increased at 1 h after EMP exposure and reached $2^{1.63}$ folds to the sham level (vs. sham group, $P < 0.05$), then further increased and reached $2^{3.21}$ folds (vs. sham group, $P < 0.05$) to the sham level at 2 h after EMP exposure. The TGF- β 3 mRNA level decreased at 6 h after EMP exposure and reached $2^{-1.74}$ folds to the sham level.

4. Discussion

The potential effects of EMP exposure on male reproductive system and fertility have attracted public attention in recent years. A survey of published scientific literature indicated that exposure to EMP could induce a large range of health problems including: dysfunction of BTB, decrease the secretion of gonadotropic hormone (GnRH), fertility, congenital malformations, perinatal death, intrauterine dysplasia; sexual dysfunction, abnormal embryonic development, etc. (Knve, 2001; Nordstom et al., 1983; Schastnyĭ et al., 1996; Zagorskata and Rodina, 1990). In this study we found that exposure to EMP-induced AsAb production in mice. AsAb has been shown to be the key factor affecting the reproductive activity in males (Francavilla et al., 2007). Among the factors causing infertility, 30% are related to immunity (Naz, 2004). In addition, it is suggested that BTB disruption is one of important reasons of AsAb production. Considering our previous work that EMP-induced an increase in BTB permeability, we presume that AsAb production is resulted from BTB disruption after EMP exposure.

To explore the mechanism of EMP-induced BTB permeability change, we observed the expression of BTB tight-junction (TJ)-associated proteins such as ZO-1 and Occludin. TJ is an important structure of BTB, which plays a vital role in maintaining the function of BTB. ZO-1 is an intracytoplasmic protein that combines TJ membrane protein Occludin and the cytoskeleton. Thus, TJ membrane protein and the cytoskeleton are coupled and further correlate with the degree of tightness of TJs, thereby supporting cell formation. Occludin is a single-strand polypeptide, with a relative molecular weight of 60–65 kDa, in the TJ membrane integral protein—an intracellular junction molecule that depends on Ca^{2+} and performs defensive and barrier functions (Galli and Barbui, 2005). It was reported that changes in TJ protein expression, posttranslational modification, subcellular localization, and protein–protein interactions were associated with increased BTB permeability (Wong et al., 2010). In this study, we focused on the ‘possible’ alterations of TJ proteins as mechanism(s) involved in BTB disruption after EMP exposure. Both immunofluorescence and western blot data showed that the expression of ZO-1 but not Occludin decreased following

EMP exposure. It was reported that alterations in Occludin, ZO-1, and ZO-2 protein localization during hypoxia and post-hypoxic reoxygenation correlated with the changes in blood–brain barrier (BBB) permeability (Mark and Davis, 2002). In our study, the permeability of BTB increased after EMP exposure concurrently with decreased ZO-1 protein level, which suggest that the alteration in protein level of ZO-1 was involved in EMP-induced BTB disruption. The expression level of Occludin did not change after EMP exposure, probably it was the alteration in Occludin protein localization but not the protein level was involved in EMP-induced BTB permeability increase, to confirm this hypothesis, further research is needed.

Vimentin constitutes the cytoskeleton of Sertoli cells and plays a vital role in sperm formation (Snásel et al., 2000), which is distributed around the nucleus and perform important functions in intracellular and extracellular activities, including maintaining Sertoli cell form, sperm secretion, etc. It was reported that alteration in intracellular vimentin may result in changes in Sertoli cell form and can thus induce TJ changes among Sertoli cells and lead to changes in BTB permeability (Bomont et al., 2000). Therefore, we observed the distribution and expression of vimentin in Sertoli cells. It was found that the distribution but not the expression of vimentin changed after EMP exposure, and vimentin change was closely related with intensity and pulse number of EMP. Usually, under normal conditions, the vimentin is mainly distributed around the cell and nucleus. After the action of mechanical force, the key to cell morphology changes is cytoskeleton reconstruction; this is mainly due to the fact that both the distribution and content of the cytoskeleton are modified. The reconstruction of the cytoskeleton is the adaptation targeting the mechanical environment, and the connection between the cytoskeleton and transmembrane molecules is an important link for transmembrane force transmission (White et al., 1983). Our results suggested that the alteration in vimentin distribution was also involved in EMP-induced BTB disruption.

TGF- β 3 is an important regulating factor of Sertoli cell TJ kinetics (Lui et al., 2001). In vitro models, TGF- β 3 has been developed to evaluate changes in target gene expression during Sertoli cell TJ assembly, and it has been found that this process is associated with a temporary decrease in both the mRNA and protein levels of TGF- β 3 (Konrad et al., 2006). Additionally, recombinant TGF- β 3 may interfere with well-formed Sertoli cell TJ barrier in vitro (Lui et al., 2003). Regarding the study on physiological correlation between in vivo and in vitro models, it has been found that BTB damage induced by CdCl₂ correlates with an increase in both mRNA and protein levels of TGF- β 3 (Wong et al., 2004). All these results demonstrate that TGF- β 3 is an important cell factor for regulating Sertoli cell TJ kinetics (Lui et al., 2003). To investigate the role of TGF- β 3 in EMP-induced Sertoli cell TJ damage, we observed the mRNA level of TGF- β 3. The results showed that the TGF- β 3 mRNA level increased in mouse testicular tissue at 1 h and 2 h after EMP exposure. In addition, the protein level of ZO-1 decreased at the corresponding time points, therefore, these results support a possibility by which the increased level of TGF- β 3 mRNA decreases the expression of ZO-1 protein and thus affects BTB permeability.

It was reported that the increase in BTB permeability is closely correlated with the expression level of TJ-associated proteins, TGF- β 3, and vimentin and free radical content (Siu et al., 2009). In this study, the alteration of TGF- β 3 mRNA level, ZO-1 protein level, as well as the structure and morphology of vimentin in Sertoli cells were found after EMP exposure, these may be the part of the mechanism via which EMP results in increased BTB permeability. Although the intensity of EMP in this study is much more higher than actual EMP levels in some occupational conditions, more attention should be paid on the biological effects of such kind of signal and further work is needed.

In conclusion, EMP exposure may increase the permeability in BTB through TGF- β 3 pathway by lowering the expression of ZO-1, re-localization of vimentin and finally resulted in AsAb production.

Conflict of interest statement

There are none.

Acknowledgments

This work was supported by National Natural Science Foundation of China (Nos. 60871068 and 30970670), and Natural Science Foundation of Shaanxi Province (No. C267).

References

- Baste, V., Riise, T., Moen, B.E., 2008. Radiofrequency electromagnetic fields; male infertility and sex ratio of offspring. *Eur. J. Epidemiol.* 23 (5), 369–377.
- Bohring, C., Krause, W., 2005. The role of antisperm antibodies during fertilization and for immunological infertility. *Chem. Immunol. Allergy* 88, 15–26.
- Bomont, P.C.L., Blondeau, F., Ben, H.C., Belal, S., Tazir, M.D.E., Topaloglu, H., Korinthenberg, R., Tüysüz, B., Landrieu, P., Hentati, F., Koenig, M., 2000. The gene encoding gigaxonin, a new member of the cytoskeletal BTB/kelch repeat family, is mutated in giant axonal neuropathy. *Nat. Genet.* 26 (3), 370–374.
- Cantz, T., Jochheim, A., Cieslak, A., Hillemann, T., Scharf, J., Manns, M.P., Ott, M., 2003. PCR-based quantification of amplified RNA from laser microdissected mouse liver samples. *Exp. Mol. Pathol.* 75 (1), 53–57.
- Chen, Y.B., Li, J., Qi, Y., Miao, X., Zhou, Y., Ren, D., Guo, G.Z., 2010. The effects of electromagnetic pulses (EMP) on the bioactivity of insulin and a preliminary study of mechanism. *Int. J. Radiat. Biol.* 86 (1), 22–26.
- Cheng, C.Y., Mather, J.P., Byer, A.L., Bardin, C.W., 1986. Identification of hormonally responsive proteins in primary Sertoli cell culture medium by anion-exchange high performance liquid chromatography. *Endocrinology* 118 (2), 480–488.
- Dimitrova, D.K., Kalaïdzhiev, S.K., Nakov, L.S., 2002. Methods for the detection of antisperm antibodies associated with immunologically-mediated human infertility. *Akush. Ginekol. (Sofia)* 41 (3), 43–48.
- Francavilla, F., Santucci, R., Barbonetti, A., Francavilla, S., 2007. Naturally-occurring antisperm antibodies in men: interference with fertility and clinical implications. An update. *Front. Biosci.* 1 (12), 2890–2911.
- Galli, M., Barbui, T., 2005. Antiphospholipid syndrome: clinical and diagnostic utility of laboratory tests. *Semin. Thromb. Hemost.* 31 (1), 17–24.
- Grima, J., Zhu, L.J., Cheng, C.Y., 1997. Testis is tightly associated with testicular cell membrane upon its secretion by Sertoli cells whose steady-state mRNA level in the testis correlates with the turnover and integrity of inter-testicular cell junctions. *J. Biol. Chem.* 272 (10), 6499–6509.
- ICNIRP Guidelines, 1998. Guidelines for limiting exposure to time-varying electric, magnetic, and electromagnetic fields. *Health Phys.* 74 (4), 494–522.
- IEEE-SA Standards Board, 1999. IEEE Standard for Safety Levels with Respect to Human Exposure to Radio Frequency Electromagnetic Fields, 3 kHz to 300 GHz. Printed in the United States of America, p. 8.
- Jiang, Y., Jahagirdar, B.N., Reinhardt, R.L., Schwartz, R.E., Keene, C.D., Ortiz-Gonzalez, X.R., Reyes, M., Lenwik, T., Lund, T., Blackstad, M., Du, J., Aldrich, S., Lisberg, A., Low, W.C., Largaespada, D.A., Verfaillie, C.M., 2002. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 418 (6893), 41–49.
- Knve, B., 2001. Electromagnetic fields and health outcomes. *Ann. Acad. Med. Singapore* 30 (5), 489–493.
- Konrad, L., Luers, G.H., Volck-Badouin, E., Keilani, M.M., Laible, L., Aumuller, G., Hofmann, R., 2006. Analysis of the mRNA expression of the TGF-Beta family in testicular cells and localization of the splice variant TGF-beta2B in testis. *Mol. Reprod. Dev.* 73 (10), 1211–1220.
- Li, D.K., Odouli, R., Wi, S., Janevic, T., Golditch, I., Bracken, T.D., Senior, R., Rankin, R., Iriye, R., 2002. A population-based prospective cohort study of personal exposure to magnetic fields during pregnancy and the risk of miscarriage. *Epidemiology* 13 (1), 9–20.
- Lipshultz, L.I., Murthy, L., Tindall, D.J., 1982. Characterization of human Sertoli cells in vitro. *J. Clin. Endocrinol. Metab.* 55 (2), 228–237.
- Lui, W.Y., Lee, W.M., Cheng, C.Y., 2001. Transforming growth factor-b3 (TGFb3) perturbs the inter-Sertoli tight junction permeability barrier in vitro possibly mediated its effects on occludin, zonula occludens-1, and claudin-11. *Endocrinology* 142 (5), 1865–1877.
- Lui, W.Y., Lee, W.M., Cheng, C.Y., 2003. TGF-betas: their role in testicular function and Sertoli cell tight junction dynamics. *Int. J. Androl.* 26 (3), 147–160.
- Mark, K.S., Davis, T.P., 2002. Cerebral microvascular changes in permeability and tight junctions induced by hypoxia-reoxygenation. *Am. J. Physiol. Heart Circ. Physiol.* 282 (4), H1485–H1494.
- Naz, R.K., 2004. Modalities for treatment of antisperm antibody mediated infertility: novel perspectives. *AJRI* 51 (5), 390–397.
- Nisarg, R.D., Kavindra, K.K., Ashok, A., 2009. Pathophysiology of cell phone radiation: oxidative stress and carcinogenesis with focus on male reproductive system. *Reprod. Biol. Endocrinol.* 22 (7), 114–118.
- Nordstom, S., Birke, E., Gustavsson, L., 1983. Reproductive hazards among workers at high voltage substations. *Bioelectromagnetics* 24 (11), 91–101.
- Schastnyĭ, S.A., Shchukin, S.I., Roslyĭ, I.M., Zubenko, V.G., Beliaev, K.R., Semikin, G.L., Morozov, A.A., 1996. Mechanism of action of electromagnetic fields biologically adequate to man. *Vestn. Ross Akad. Med. Nauk.* (5), 51–54.
- Siu, E.R., Wong, E.W., Mruk, D.D., Sze, K.L., Porto, C.S., Cheng, C.Y., 2009. An occludin-focal adhesion kinase protein complex at the blood–testis barrier: a study using the cadmium model. *Endocrinology* 150 (7), 3336–3344.
- Slama, R., Ducot, B., Keiding, N., Bouyer, J., 2004. Studying human fertility and environmental exposures. *Environ. Health Perspect.* 112 (11), A604–A606.
- Snásel, J., Shoeman, R., Horejsĭ, M., Hrusková-Heidingsfeldová, O., Sedláček, J., Ruml, T., Pichová, I., 2000. Cleavage of vimentin by different retroviral proteases. *Arch. Biochem. Biophys.* 377 (2), 241–245.
- Stern, J.E., Nelson, T.S., Gibson, S.H., Colby, E., 1994. Antisperm antibodies in female mice: responses following intrauterine immunization. *Am. J. Reprod. Immunol.* 31 (4), 211–218.
- Wang, X.W., Ding, G.R., Shi, C.H., Zhao, T., Zhang, J., Zeng, L.H., Guo, G.Z., 2008. Effect of electromagnetic pulse exposure on permeability of blood–testicle barrier in mice. *Biomed. Environ. Sci.* 21 (3), 218–221.
- White, G.E., Gimbrone, M.A., Fujiwara, K., 1983. Factors influencing the expression of stress fibers in vascular endothelial cells in side. *J. Cell Biol.* 97 (2), 416–424.
- Wong, C.H., Mruk, D.D., Lui, W.Y., Cheng, C.Y., 2004. Regulation of blood–testis barrier dynamics: an in vivo study. *J. Cell Sci.* 117 (Pt 5), 783–798.
- Wong, E.W., Mruk, D.D., Lee, W.M., Cheng, C.Y., 2010. Regulation of blood–testis barrier dynamics by TGF-beta3 is a Cdc42-dependent protein trafficking event. *Proc. Natl. Acad. Sci. U.S.A.* (Epub ahead of print).
- Zagorskata, E.A., Rodina, G.P., 1990. Reaction of the endocrine system and peripheral blood of rats to a single and chronic exposure to pulsed low frequency electromagnetic field. *Kosm-Bio-Aviakosm-Med.* 24 (2), 56–60.