

Study on Pulsed Electromagnetic Fields Regulate the mRNA Expression of V-ATPase, CAII, NFAT2 and RANK in an Ovariectomized Rat Model of Osteoporosis Mechanism Analysis

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Abstract

We investigated the mRNA expression of V-ATPase, CA II, NFAT2, and RANK as well as bone mineral density (BMD) in bilaterally ovariectomized (OVX) rats treated with pulsed electromagnetic fields (PEMF), to explore the mechanism by which PEMF alter bone at the molecular level *in vivo*. 24 Sprague–Dawley female rats were randomly divided into three different groups: SHAM, OVX, ovariectomy with PEMF stimulation (PEMF+OVX). The PEMF frequency was 3.8 mT, 8 Hz, for 40 min per day. Eight rats in each group were treated for 28 days. At the end of the intervention, the body weight and BMD were measured. The mRNA expression of V-ATPase, CA II, NFAT2 and RANK were analyzed by real-time fluorescent-nested quantitative polymerase chain reaction (RT-PCR). The V-ATPase, CAII, NFAT2 and RANK genes show strong expression in the OVX group as compared with the SHAM group ($P < 0.001$, respectively). The mRNAs expression of V-ATPase, CAII, NFAT2 and RANK in PEMF treatment group were significantly lower than that in OVX group, respectively ($p = 0.001$, $p = 0.000$, $p = 0.013$, $P = 0.04$, respectively). The V-ATPase, CAII and RANK mRNA expression in the PEMF+OVX group were higher than SHAM group ($p = 0.04$, $p = 0.001$, $p = 0.000$, respectively). However, the NFAT2 mRNA expression did not show a statistically significant difference between the SHAM and the PEMF+OVX group ($P = 0.08$). This study indicated that exposure to PEMF restrained bone resorption in order to elevate BMD, and this outcome occurred in part through RANK-signaling by the CN/NFAT pathway to regulate bone metabolism. Alternatively, PEMF may promote the BMD through regulating the expression of NFAT2 genes. It may have certain significance in the treatment target of osteoporosis.

Keywords: PEMF; V-ATPase; CAII; NFAT2; RANK

Introduction

Osteoporosis is a major public health problem around the world as it results in fractures with minimal trauma. It is a condition of low bone mass and microarchitectural deterioration of bone tissues. Bone is a complex tissue that continually undergoes renewal and repair throughout life, by a process termed bone remodeling. The number of bone fractures due to osteoporosis is expected to be 6.26 million in 2050. This is an increase from the 1.56 million fractures reported in 1990 [1]. Moreover, osteoporosis is more common in women, and postmenopausal osteoporosis is the most common type. The ovariectomized (OVX) rat model is widely used in the study of postmenopausal bone loss-related problems. There are many similarities between the rat and the human femur, at both the macro-structural and microstructural levels [2].

Carbonic anhydrase II (CA II) is one form of carbonic anhydrase, a family of enzymes [3]. Hydrogen ions are produced via CA II catalyzes the reversible reaction between CO_2 and H_2O in the cytoplasm and is secreted into the extracellular space by H^+ -ATPase (V-ATPase) [4]. CA II is characteristically expressed during the early stage of osteoclast differentiation [5]. However CA II plays important roles not only in bone resorption but also in osteoclast differentiation processes. While in the mature osteoclast, mRNA expression of CA II and calcitonin receptor (CTR) are increased in the resorptive state and in response to acidosis, regulating osteoclast pH^+ [6]. V-ATPase mediates proton transport to allow secretion of acid, which directly dissolves the bone matrix and activates secreted proteases that participate in bone resorption. Therefore, V-ATPases are closely related to CA II, and play an important role in bone resorption mediated by osteoclast [7,8].

Studies have revealed that nuclear factor of activated T-cells

cytoplasmic1 (NFAT2) is a critical transcription factor in osteoclastogenesis. Hirotani et al. [9] found that NFAT plays a crucial role in the differentiation of osteoclast precursor cells into mature osteoclasts. Numerous resorptive stimuli have been shown to enhance osteoclast differentiation and proliferation and to accelerate bone resorption. Asagiri et al. [10] found that NFAT2^{+/-} cells differentiate into osteoclasts with bone-resorbing activity, whereas NFAT2^{-/-} cells are unable to generate osteoclasts, despite normal development into the monocyte/macrophage lineage the formation of bone-resorbing osteoclasts was rescued by retroviral expression of NFAT2. Crabtree et al. [11] claimed that NFAT transcription complexes function as signal integrators and coincidence detectors. Currently, there are mainly four important signal transduction pathways related to Receptor Activator for Nuclear Factor- κ B Ligand (RANKL), calcineurin/nuclear factor of activated T cells (CN/NFAT) pathway is one of them [12,13].

Receptor activator of nuclear factor- κ B (RANK) and RANKL belong to a TNF superfamily receptor-ligand pair. Osteoblasts produce the osteoclast differentiation factor RANKL in response to several bone resorbing factors. Terminal differentiation of monocytes/

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Received: January 06, 2017; **Accepted:** February 13, 2017; **Published:** March 05, 2017

Citation: Huang H, Chen J, He JQ, Fu JF (2017) Study on Pulsed Electromagnetic Fields Regulate the mRNA Expression of V-ATPase, CAII, NFAT2 and RANK in an Ovariectomized Rat Model of Osteoporosis Mechanism Analysis. J Bioengineer & Biomedical Sci 7: 217. doi: [10.4172/2155-9538.1000217](https://doi.org/10.4172/2155-9538.1000217)

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macrophages into osteoclasts relies on signaling by RANKL. High Ca^{2+} alone can increase osteoclastogenesis. Further, high Ca^{2+} -induced osteoclastogenesis may be mediated by osteoblasts via up-regulation of RANKL expression [14]. Klee et al. [15] found that calcium signaling activates the phosphatase calcineurin and induces movement of NFAT2 proteins into the nucleus to form complexes on DNA. Ca^{2+} regulates calcineurin which in turn dephosphorylates and induces the nuclear localization of the cytoplasmic components (NFATc proteins) of NFAT transcription complexes [16]. RANKL stimulates release of Ca^{2+} which leads to calcineurin-mediated activation of NFAT2, triggering a sustained NFAT2-dependent transcriptional program during osteoclast differentiation [17,18]. NFAT2 thus represents a master switch regulating terminal differentiation of osteoclasts, which functions downstream of RANKL [19]. The RANK-RANKL system is essential for regulation of osteoclast differentiation *in vivo*. Disruption of RANKL or RANK results in osteopetrosis as a consequence of impaired osteoclast differentiation [20,21].

Pulsed electromagnetic fields (PEMF) may have an effect on the treatment of osteoporosis. Li et al. showed that PEMFs might be considered a factor that can affect osteogenesis of osteoblasts [22]. Further study revealed that extremely low intensity, low frequency, single pulse electromagnetic fields restore trabecular bone structure and suppress trabecular bone loss in bilateral ovariectomized rats, augmenting and restoring proximal tibial metaphyseal trabecular bone mass and architecture [23]. PEMF stimulation for 30 days restored trabecular bone mass of PEMF+OVX rats to levels equivalent to age-matched intact rats. We have previously reported that PEMFs applied at 3.8 mT, 8 Hz, for 40 min per day could regulate osteoclast gene expression of CA II and RANK *in vivo* and *in vitro* [24,25].

In spite of the accumulating evidence, the exact mechanisms by which cell adhesion signals are associated with osteoporosis have not been fully elucidated. In the present study, we attempted to clarify whether PEMF can regulate the gene expression of CAII, NFAT2, RANK and V-ATPase of osteoclast *in vivo*. The results may be used in future to provide new safety therapies for osteoporosis.

Materials and Methods

Animals

The experiments were carried out using 24 three-month-old female Sprague-Dawley rats (weighing 186 ± 6 g each) that were provided by the Medical College of Xiamen University Laboratory Animal Center (certificate number: 2007-0005), Xiamen, China. All rats were fed with diet *ad libitum*. Rats were housed in the micro-isolator system under standard conditions (temperature $22 \pm 2^\circ\text{C}$ and a humidity of 70%) with strict aseptic handling techniques enforced at all times. The experimental protocol was approved by the medical ethics committee of Zhongshan Hospital of Xiamen University and conforms to National Institutes of Health guidelines regarding animal experimentation.

Reagents and electromagnetic stimulation system

Low frequency pulsed electromagnetic treatment for osteoporosis was applied by the UNION-2000. TRIzol® was purchased from Invitrogen (Life Technologies, Carlsbad, CA, USA). Taq DNA polymerase and dNTP were from Takara Bio Group (Dalian, China). RevertAid™ first strand cDNA synthesis kits were purchased from MBI Co. (MBI Fermentas, Vilnius, Lithuania). PCR primers and probes were from Shanghai Biological Engineering (Shanghai, China).

Ovariectomy

In the first set of experiments, 24 healthy female SD rats were

randomly assigned to three groups: SHAM, OVX, PEMF+OVX. The SHAM group comprised 8 rats in which retroperitoneal fat with the same mass as bilateral ovaries was removed under general anesthesia with 5% chloral hydrate (0.6 mL/100 g). The remaining 16 rats underwent ovariectomy, performed under the same conditions of anesthesia. Twelve weeks after surgery, the 16 rats were randomly divided into two experimental groups: OVX, PEMF+OVX, 8 rats per group.

Experimental protocols

The rats in PEMF+OVX group were exposed to whole body PEMFs of 3.8 mT, 8 Hz, for 40 min per day. All rats in each group were treated daily as protocol, and sacrificed at 28 days.

Body weight and bone mineral density (BMD)

Before the endpoint of the experiment, animal's body weight was measured. After the rats were euthanized, and the left femurs were removed for measurement of BMD by dual energy X-ray absorptiometry (DEXA) (GE Healthcare, Little Chalfont, UK). The left ilium was immediately stored at -80°C for PCR.

Determination of messenger ribonucleic acid levels using Real-Time PCR

Total RNA was extracted from the left ilium of rats in each group using TRIzol® reagent (Invitrogen). Reverse transcription and polymerase chain reactions were carried out as nested PCR. The nested RT-PCR conditions were established and optimized for each primer pair. To determine the sensitivity of the nested RT-PCR, definitive amounts of transcripts synthesized from a cloned cDNA of the corresponding gene (V-ATPase, CA II, NFAT2 or RANK) were used. The synthesis of the cDNA was primed separately for detection of V-ATPase mRNA, CA II mRNA, NFAT2 mRNA and RANK mRNA. The threshold cycle (Ct) value was calculated from amplification plots [26].

The ΔCt value for each sample was obtained by subtracting the Ct values of a housekeeping gene (β -actin). Samples from the OVX, PEMF+OVX groups were included in each experiment, and each experiment was carried out at least 3 times. The sequences of reaction products were confirmed by agarose gel electrophoreses, and the control reactions run in the absence of reverse transcription did not generate any product. The expression levels of V-ATPase, CA II, NFAT2 or RANK mRNA were determined relative to the expression level of β -actin mRNA.

The specific sequences of the PCR primers for V-ATPase, CA II, NFAT2, RANK, and β -actin and the expected PCR product lengths are listed in Table 1.

Statistical analysis

The mean values of the differences between the study groups in all of the comparative bioassays were assessed using one-way ANOVA. Data analysis was performed using SPSS 16.0 software, $P < 0.05$ was considered significant.

Results

Body weight

Before experiment, there were no significant differences in body weight between the groups ($P < 0.05$), but there were significant differences between the groups at the end of the experiment ($P = 0.000$) (Figure 1).

BMD

BMD of the left femur was measured using DEXA. At each time-

Gene	5'—3'	Sequence	Size(bp)
rRANK	Forward	TCAGCATTTACTACAGGAAGGGAG	209
	Reverse	AGCACAGTCTTCTGGAACCATCT	
rV-ATPase	Forward	CACAACAGACAGATTTACCCTCCTA	180
	Reverse	GGCTTCTTCTCCACCACAG	
rNFAT2	Forward	GGAGGGAAGAAGATGGTGTGT	182
	Reverse	CTGGTTATTCTCTGGTTGCGG	
rCAII	Forward	ATCCTTGCTCCCTTCTCTCTG	201
	Reverse	GACGCCAGTTGTCCACCATC	
GAPDH	Forward	AGTGCCAGCCTCGTCTCATAG	192
	Reverse	CGTTGAACTTGCCGTGGGTAG	

Table 1: Primer sequences and PCR product length.

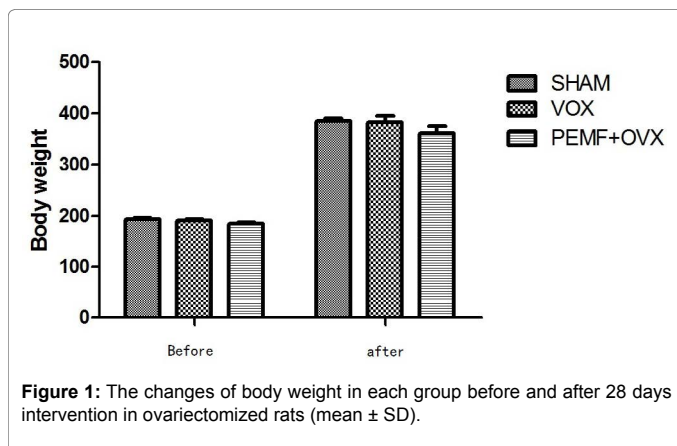


Figure 1: The changes of body weight in each group before and after 28 days intervention in ovariectomized rats (mean \pm SD).

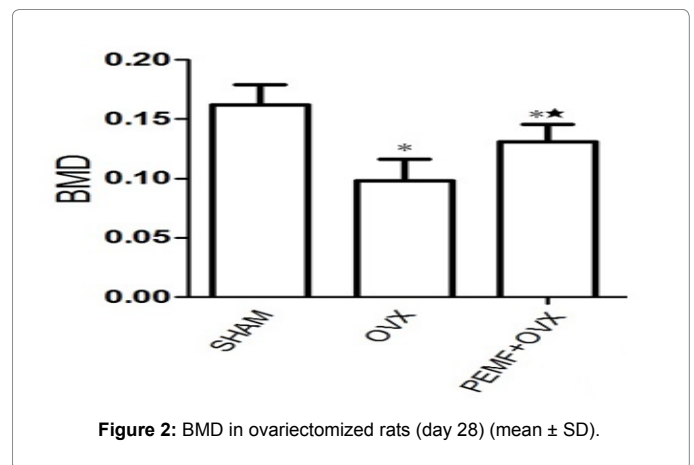


Figure 2: BMD in ovariectomized rats (day 28) (mean \pm SD).

point, BMD was highest in the SHAM group (day 28: 0.156 ± 0.019), and lowest in the OVX group (day 28: 0.050 ± 0.014). There were significant differences between each of the groups ($P < 0.05$) (Figure 2).

*Compared to the SHAM group $P < 0.05$

★Compared to the OVX group $P < 0.05$

mRNA expression of V-ATPase, CA II, NFAT2 and RANK in each group

RT-PCR was used to analyze mRNA expression of V-ATPase, RANK, CA II and NFAT2 for all rats on day 28 (Figure 3).

At the end of the experiment, we found significant differences in mRNA expression of V-ATPase, RANK, CA II and NFAT2 between all the groups after 28 days.

As shown in Figure 3, the four genes show strong expression in the OVX group as compared with the SHAM group respectively ($P < 0.001$, respectively). The mRNA expression of V-ATPase, CAII, NFAT2 and RANK in PEMF treatment group were significantly lower than that in OVX group, respectively ($p = 0.001$, $p = 0.000$, $p = 0.013$, $P = 0.04$, respectively).

The V-ATPase, CAII and RANK mRNA expression in the PEMF+OVX group were higher than SHAM group ($p = 0.04$, $p = 0.001$, $p = 0.000$, respectively). However, the NFAT2 mRNA expression did not show a statistically significant difference between the SHAM and the PEMF+OVX group ($P = 0.08$).

* Compared to the SHAM group $P < 0.05$

★ Compared to the OVX group $P < 0.05$

Discussion

An imbalance between osteoclasts and osteoblasts may lead to

various bone-related disorders such as osteoporosis, osteomalacia, and osteopetrosis [27,28]. The ovariectomized (OVX) rat is a well-proven animal model for osteoporosis studies [29]. Rats can be ovariectomized to make them sex-hormone deficient, and thus simulate the accelerated loss of bone that occurs in women following menopause. Ovariectomy-induced bone loss in the rat and postmenopausal bone loss share many similar characteristics [30].

RANK is a type I transmembrane protein originally cloned from dendritic cells [31], which is expressed by cells of the monocyte/macrophage lineage. RANKL stimulates osteoclastogenesis and osteoclast activity by binding to the cell surface receptor RANK, located on osteoclasts [32,33]. Two key cytokines, M-CSF and RANKL, are necessary and sufficient for osteoclast differentiation and activation. RANKL signaling in osteoclast precursor cells also affects the phosphoinositide kinase-3/Akt, NFAT2, activator protein 1 (AP-1), and nuclear factor kappa B (NF- κ -B) pathways [34,35]. These signaling pathways ultimately lead to induction and activation of the transcription factors involved in the expression of genes that characterize osteoclasts.

NFAT is a calcineurin and calcium-regulated transcription factor. The release of Ca^{2+} activates the calmodulin-regulated phosphatase calcineurin, which binds the N-terminal domain of NFAT2 and dephosphorylates it [19]. NFAT2-deficient embryonic stem cells fail to differentiate into osteoclasts in response to RANKL stimulation, and the ectopic expression of NFAT2 causes precursor cells to undergo efficient differentiation without RANKL signaling. Ogawa et al. [35] found that L-serine plays a crucial role in the expression of NFAT2 in RANKL-induced osteoclast formation *in vitro*. Together with a study reported by Song et al. their data showed that NFAT2 was a key regulator of osteoclast migration and adhesion to the bone surface through

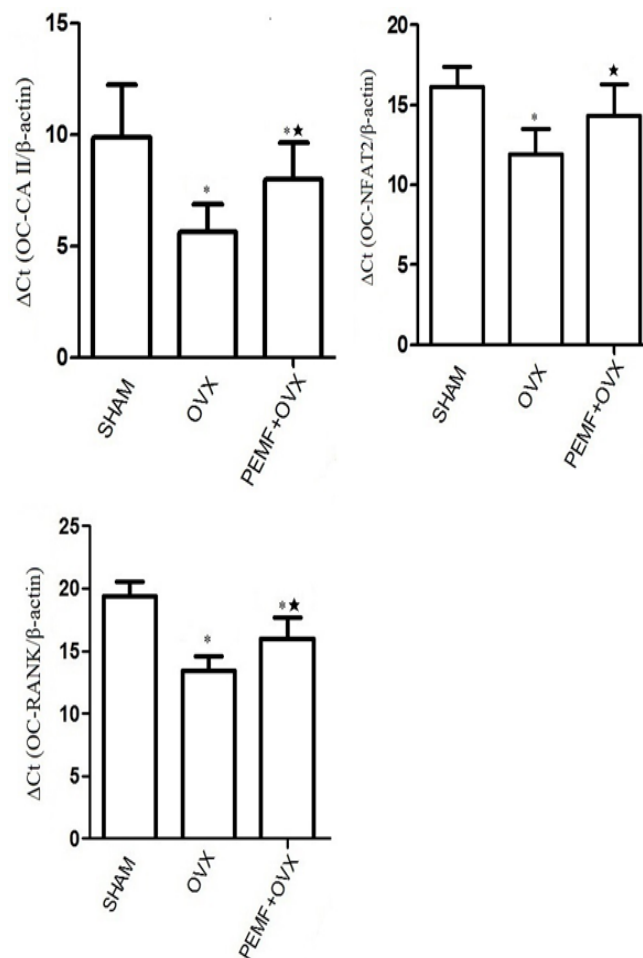


Figure 3: mRNA expressions in each group after 28 days in respectively were assessed by RT-PCR.

induction of $\beta 3$ integrin and c-Src. c-Src is essential to the function of osteoclasts, but not to osteoclast formation [36]. Osteoclasts deficient for c-Src exhibit reduced motility and abnormal organization of the ruffled border, do not develop normal podosomes, lack the cytoskeletal elements and are unable to give rise to mature superstructures, and fail to spread properly [37]. It has been concluded that NFAT2 is not only sufficient but also indispensable for osteoclastogenesis.

CA II present in the osteoclast cytoplasm accompanies variations of osteoclast activity. It appears that high gene expression during the bone resorbing period affects proliferation and activity of osteoclasts. CA II catalyzes the reaction between CO_2 and H_2O to synthesize H_2CO_3 , which then dissociates into H^+ and HCO_3^- . The process of acid demineralization in osteoclasts is accomplished through V-ATPase, which pumps hydrogen ions formed and released by specific CA II into the extracellular compartment. It generates the H^+ required for osteoclast-mediated bone resorption in humans. It is highly expressed on ruffled borders of the plasma membrane of functional osteoclasts, and it is essential for bone degradation prior to resorption. Patients with genetic defects in V-ATPase isoforms that exist in osteoclast plasma membranes develop osteopetrosis [38]. Thus, V-ATPase is being investigated as a potential drug target in treating osteoporosis characterized by excessive bone loss. It has also been reported that the

expression of CA II, cathepsin K, and MMP-9 in RAW264.7 cells was not induced by M-CSF, but by RANKL in the presence of interleukin- 1α [39]. Another gene that closely linked to osteoclastogenesis is V-ATPase, it is ATP-dependent proton pump that divided into intracellular and plasma membrane according to the type of cells. Thus, V-ATPase is being investigated as potential drug targets in treating osteoporosis, which is characterized by excessive bone loss.

PEMF as a tool for clinical therapy, widely used for patients with delayed fracture healing or non-unions [40,41]. As shown by clinical evidence, therapeutic effects can be achieved for union-delayed and nonunion bone fractures using PEMF treatment at any fracture location. As we previously reported [25], PEMF reduces the gene expression of RANK, CA II and NAFT2 *in vitro*. A study found PEMF affect only the osteoclast phenotype after short exposure times [42]. It has been determined that electric stimulation can enhance osteoblast differentiation [43], increase osteoblast proliferation and consequently increase bone formation [44,45]. The mechanism or PEMF to inhibit bone loss is not well understood. Even though some findings have been reported that suggest that PEMF affect osteoclasts *in vitro*, experiments to understand the effect of PEMF on osteoclasts *in vivo* is still required.

In a previous study, it has been reported that PEMF applied at 3.8

mT, 8 Hz could regulate the gene expression of CAII in ovariectomized rats. V-ATPase is associated with CAII and foretells the process of osteoclastic bone resorption. The V-ATPase result is in accordance with these preliminary reports. From these data it is inferred that PEMF restrain bone resorption by RANK-signaling of the CN/NFAT pathway. Our results showed that mRNA expression of V-ATPase, CA II, NFAT2, RANK in the PEMF group decreased in the PEMF+OVX group after 28 days treatment ($P < 0.05$), while the mRNA expression of NFAT2 were equivalent between the PEMF+OVX group and the SHAM group after intervention ($P > 0.05$).

It was interesting to find that at the end of the experiment, there were no significant differences between the SHAM and PEMF+OVX groups with regard to expression of NFAT2 mRNA ($P > 0.05$). We can speculate that PEMF may permote the BMD through regulating the expression of NFAT2 genes.

BMD is an important indicator of quantitative bone mass and metabolism. Using DEXA to measure BMD is considered to be the gold standard for diagnosis of osteoporosis [46]. DEXA scanning also provides high precision and accuracy for evaluating the BMD of rats *in vivo* and *in vitro* [47]. Zhou et al. reported that PEMF could increase serum E_2 levels and BMD in OVX rats [48]. The result in our study showed that BMD in the SHAM group was significantly increased above the BMD of the OVX group. Moreover, BMD were higher in the PEMF group than in the OVX group after 28 days. These findings suggest PEMF have a role in positively adjusting bone metabolism after 28 days of intervention.

The specific mechanism regulating treatment-related genes is not clear and requires further investigation.

Conclusion

In conclusion, our data demonstrate that PEMF regulate the gene expression of V-ATPase, RANK, CA II and NFAT2. Therefore, on the basis of our predecessors' investigations and our own findings, we infer that PEMF might modulate the process of osteoclastogenesis and subsequent bone resorption, at least partially, through V-ATPase, RANK, CA II and NFAT2. Providing a new method for the treatment of senile osteoporosis.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No. 81272168) and Medical Innovation topics of Fujian (NO. 2012-CXB-32).

Funding: This work was supported by the National Natural Science Foundation of China (No. 81272168) and the National Natural Science Foundation of Fujian (NO. 2016J01623).

Conflicts of Interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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