Puerarin, an isoflavone compound extracted from Gegen (Radix Puerariae Lobatae), modulates sclera remodeling caused by extremely low frequency electromagnetic fields

Tian Tian, Cai Xiaojing, Zhu Huang

Tian Tian, Cai Xiaojing, Zhu Huang, Department of Ophthalmology, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China

Supported by the General Program of the Bio-medical Division of the Shanghai Science and Technology Commission: Effect of Extremely Low Frequency Electromagnetic Fields on Myopia Based on RPE-sclera Complex (No.10411966200); the Scientific Research Fund of Chinese Medical of Shanghai Health Bureau: Effect of Puerarin on RPE Cells Exposed to ELF-EMF (No. 2014 JP015)

Correspondence to: Prof. Zhu Huang, Department of Ophthalmology, Xinhua Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200092, China. drzhuhuang@163.com

Telephone: +86-21-25076874
Accepted: November 15, 2015

Abstract

OBJECTIVE: To evaluate the protective effect of puerarin [an isoflavone compound extracted from Gegen (Radix Puerariae Lobatae)] in scleral remodeling induced by extremely low frequency electromagnetic fields (ELF-EMFs).

METHODS: Human fetal scleral fibroblasts (HFSFs) were divided into 5 groups: (a) untreated controls; (b) cells treated with ELF-EMFs; (c) cells treated with ELF-EMFs and puerarin 0.1 μM; (d) cells treated with ELF-EMFs and puerarin 1 μM; (e) cells treated with ELF-EMFs and puerarin 10 μM. Cell proliferation activity was measured by the cell-counting kit-8 assay. Matrix metalloproteinase-2 (MMP-2) activity was measured by gelatin enzymography. MMP-2 and collagen I (COL1A1) mRNA, protein expression were measured by Real-Time polymerase chain reaction, Western blot analysis, respectively.

RESULTS: Puerarin reduced the inhibition in cell proliferation, MMP-2 activity, mRNA, protein expression of HFSFs exposed to ELF-EMF and enhanced the COL1A1 mRNA and protein expression.

CONCLUSION: Puerarin was found to participate in the matrix remodeling process. It might be a potential agent for the treatment of extracellular matrix degradation of sclera associated with ocular conditions.

© 2016 JTCM. All rights reserved.

Key words: Puerarin; Pueraria lobata; Sclera Fibroblast; Matrix metalloproteinase 2; Collagen type I; Electromagnetic fields

INTRODUCTION

The sclera is now known to be a dynamic tissue that undergoes constant remodeling throughout life. Results from research over the last 25 years have established that sclera remodeling is regulated by genetic and environmental influences which have profound effects on ocular size and refraction. Extremely low frequency electromagnetic fields (ELF-EMFs) has been common in daily life all over the world. Both epidemiological and laboratory studies have assessed the biological effects of ELF electromagnetic field and explored its relationship with many diseases. We found that ELF-EMFs decreased collagen I (COL1A1) expression, increased Matrix metalloproteinase-2 (MMP-2) expression in Human fetal scleral fibroblasts (HFSFs). We suggested that ELF-EMFs might contribute to...
scleral remodeling, which underlies some ocular conditions such as myopia.\textsuperscript{5,6}

Puerarin is the main bioactive ingredient derived from the root of the Pueraria lobata (Willd.) Ohwi, widely known as Gegen (\textit{Radix Puerariae Lobatae}) in Traditional Chinese Medicine (TCM).\textsuperscript{7} Pharmacological researches of puerarin in various diseases have made great progress.\textsuperscript{8,9} Studies showed that it can regulate matrix metabolism,\textsuperscript{10,11} but the effects of puerarin on protecting sclera pathological remodeling caused by ELF-EMFs were less reported. In this paper we detected the effect of puerarin on cell viability, the synthesis and secretion of cytokines, and enzymes in HFSFs.

**MATERIALS AND METHODS**

**Exposure system**

Helmholtz coils (16 cm length, 14 cm width, 8 cm height and 168 turns copper wire each) were designed to generate an effective magnetic field in the range of 0-2 mT, with a sinusoidal wave of 50 Hz. The magnetic flux density at the center of coil was measured by an electromagnetic field radiation tester (EMF-827, Lutron Electronic Enterprise Co., Ltd., Taiwan, China). The exposure system was put in a humidified incubator at 37 °C with 5% CO\textsubscript{2}.\textsuperscript{9}

**Cell culture and treatment**

HFSFs were obtained from Beijing Institute of Ophthalmology (Beijing, China). Cells were cultured in Dulbecco’s modified Eagle’s medium with penicillin and streptomycin and 10% fetal bovine serum (Gibco BRL, Gaithersburg, MD, USA), incubated at 37 °C in a humidified incubator containing 5% CO\textsubscript{2}. After trypan
disin, cells were allowed to attach to the substrate for 12 h; the medium was renewed; puerarin, final concentrations of 0, 0.1, 1 and 10 μM, were added in the puerarin groups. Cells of radiation and puerarin groups were placed inside the coils with a sinusoidal 50 Hz electromagnetic field at 0.2 mT for 24 h. Control cells (not exposed to ELF-EMFs) were cultured in another incubator without power coils.

**Plant materials**

Puerarin powder (Identification of product, 110752-201313) was purchased from the control of pharmaceutical and biological products Chinese (Nanjing, China).

**Cell proliferation assay**

The cells were cultured at initiated number of 6000 cells/well. The rate of cell proliferation was estimated using the cell counting kit-8 (CCK-8) assay (Dojindo, Laboratorie Kumamoto, Japan), which was performed according to the manufacturer’s protocol. Briefly, cells grown in a 96-well plate were incubated with the CCK-8 solutions for 1 hr at 37 °C, following which the absorbance of each well at 450 nm was recorded.

**RESULTS**

**Cell proliferation assay**

In order to examine the effect of ELF-EMFs on HFSFs...
viability, the number of live cells growing under 50 Hz sinusoidal ELF-EMFs at the intensity of 0.2 mT were measured at 6, 12, 24 and 48 h after the exposure (Figure 1). Inhibition in cell proliferation began obviously after exposed to magnetic field for 12 hrs compared by corresponding control group (r = 3.311, P < 0.05). By 48 h, a 22.49% of reduction in total live cell number was observed compared with control group (r = 11.906, P < 0.01). In order to examine the effect of puerarin on HFSF viability treated by ELF-EMFs, cells with puerarin at final concentrations of 0, 0.1, 1 and 10 μM were grown under ELF-EMFs for 24 h (F = 77.589, P < 0.01) Puerarin at the concentration of 1.0 μM and 10 μM enhanced the proliferation activity of HFSFs compared with radiation group with puerarin at 0 μM (Figure 2).

**Figure 1** Effect of ELF-EMFs on HFSF cell proliferation
Control: cells were cultured 6, 12, 24 and 48 h without ELF-EMFs. Radiation: cells were cultured 6, 12, 24 and 48 h with ELF-EMFs. ELF-EMFs: extremely low frequency electromagnetic fields; HFSF: human fetal scleral fibroblasts. Compared to control, *P* < 0.05, **P** < 0.01.

**Figure 2** Effect of puerarin on HFSF cell proliferation treated by ELF-EMFs
1: untreated controls; 2: cells treated with ELF-EMFs; 3: cells treated with ELF-EMFs and puerarin 0.1 μM; 4: cells treated with ELF-EMFs and puerarin 1 μM; 5: cells treated with ELF-EMFs and puerarin 10 μM. ELF-EMFs: extremely low frequency electromagnetic fields; HFSF: human fetal scleral fibroblasts; ELF-EMFs: extremely low frequency electromagnetic fields. Compared with group 2, **P** < 0.01.

**Gelatin zymography analysis of MMP-2 enzyme activity**
MMP-2 enzyme activity plays essential role in regulating extracellular matrix (ECM) degradation. Therefore, how puerarin affects the activity of MMP-2 in HFSFs exposed to ELF-EMFs becomes important. MMP-2 enzyme activity was assayed from collected after 24 h. Activated forms of MMP-2 migrate to positions of 62 kDa. Electromagnetic fields increased MMP-2 enzyme activity in HFSF by 25% compared to the control. The activity of MMP-2 gradually decreased in the presence of puerarin with increasing concentrations (Figures 3, 4).

**Quantitative real-time PCR analysis of MMP-2, COL1A1**
The RQ values of MMP-2 mRNA expression in HFSFs after exposure to ELF-EMFs for 24 h at 0.2 mT flux density treated with 0, 0.1, 1 and 10 μM puerarin were 1.737 ± 0.031, 1.607 ± 0.021, 1.233 ± 0.058 and 0.953 ± 0.072, respectively. MMP-2 mRNA expression with 0μM puerarin increased by 73.7% compared to the control group (P < 0.01). MMP-2 mRNA expression gradually decreased in the presence of puerarin with increasing concentrations (Figure 5).

**Figure 3** Micrograph of MMP-2 enzyme activity analysis
1: untreated controls; 2: cells treated with ELF-EMFs; 3: cells treated with ELF-EMFs and puerarin 0.1 μM; 4: cells treated with ELF-EMFs and puerarin 1 μM; 5: cells treated with ELF-EMFs and puerarin 10 μM. ELF-EMFs: extremely low frequency electromagnetic fields; MMP-2: matrix metalloproteinase-2.

**Figure 4** Densitometric analysis of the active-MMP-2 band
1: untreated controls; 2: cells treated with ELF-EMFs; 3: cells treated with ELF-EMFs and puerarin 0.1 μM; 4: cells treated with ELF-EMFs and puerarin 1 μM; 5: cells treated with ELF-EMFs and puerarin 10 μM. ELF-EMFs: extremely low frequency electromagnetic fields; MMP-2: matrix metalloproteinase-2. Compared with group 2, **P** < 0.01.

**Figure 5** Densitometric analysis of the MMP-2 mRNA expression
1: untreated controls; 2: cells treated with ELF-EMFs; 3: cells treated with ELF-EMFs and puerarin 0.1 μM; 4: cells treated with ELF-EMFs and puerarin 1 μM; 5: cells treated with ELF-EMFs and puerarin 10 μM. ELF-EMFs: extremely low frequency electromagnetic fields; MMP-2: matrix metalloproteinase-2. Compared with group 2, *P* < 0.01 and **P** < 0.05.

The RQ values of COL1A1 mRNA expression in HFSFs after exposure to ELF-EMFs for 24 h at 0.2 mT flux density treated with 0, 0.1, 1 and 10 μM puerarin
were 0.66 ± 0.066, 0.717 ± 0.031, 0.883 ± 0.057 and 0.997 ± 0.100, respectively. COL1A1 mRNA expression with 0 μM puerarin decreased by 34% compared to the control group (P < 0.01). COL1A1 mRNA expression gradually increased in the presence of puerarin with increasing concentrations (Figure 6).

**DISCUSSION**

The sclera is a dense connective tissue that defines ocular size and shape, which biomechanical properties are determined by its ECM. Defects in scleral ECM remodeling lead to some ocular conditions such as myopia, characterized by increment in activity and expression of matrix metalloproteinases as well as the reduction of collagen.

In previous studies, it can be seen that ELF-EMFs act as a kind of stimulus causing the matrix remodeling in HFSFs. Puerarin has the role of lowering intraocular pressure, improving microcirculation and antioxidation. Puerarin injection and other preparations have been extensively used in the clinic in China. Puerarin has also been found to affect cell proliferation activity, regulate matrix metabolism, and the synthesis and secretion of cytokines and enzymes. Wang et al confirmed that 0.001 μM puerarin could decrease MMP-9 expression in endometriosis tissue. Puerarin increased the rate of proliferation and mRNA levels of type I collagen. Puerarin also has some hormone-like activities and has protective effect against oxidative stress response and osteoporosis. We have found that puerarin decreased the expression of MMP-2 mRNA and protein in HFSF cells exposed to ELF-EMFs, but whether puerarin has effect on HFSF’s cell viability, MMP-2 activity and collagen I synthesis has not yet been reported. It can be seen that puerarin has effect in preventing matrix remodeling caused by harmful stimulus. By 48 h, a 22.49% of reduction in total live cell number was observed. Puerarin at the concentration of 1.0 μM and 10 μM enhanced the proliferation activity of HFSFs compared with radiation group with puerarin at 0 μM. MMP-2 activity, mRNA and protein expres-
sion increased by 1.25, 1.737 and 1.06 folds in HFSFs exposed to ELF-EMFs compared with the control group. While COL1A1 mRNA and protein expression decreased 34% and 90% in HFSFs exposed to ELF-EMFs compared with the control group. Puerarin weakened these changes to a certain extent. In sum, our study showed that ELF-EMFs had a direct effect on cell viability, synthesis and secretion of cytokines in HFSFs. These effects can be partially reversed by puerarin. These findings suggested a potential option for the treatment of extracellular matrix degradation of sclera associated with various ocular conditions.

REFERENCES