

# GENES UNDERLYING POSITIVE INFLUENCE OF PRENATAL ENVIRONMENTAL ENRICHMENT AND NEGATIVE INFLUENCE OF PRENATAL EARTHQUAKE SIMULATION AND CORRECTIVE INFLUENCE OF CHINESE HERBAL MEDICINE ON RAT OFFSPRING: IRF7 AND NINJ2

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## Abstract

**Background:** Prenatal environmental enrichment (EE) has been proven to positively affect but prenatal stress negatively influence the physiological and psychological processes in animals, whose trans-generational genetic mechanism remains unclearly defined. We aimed to investigate and find out key genes underlying the positive-negative effects derived from prenatal interventions.

**Materials and Methods:** Pregnant rats were randomized into EE group (EEG), earthquake simulation group (ESG), herbal group (HG) received herbal supplements in feed after earthquake simulation, and control group (CG).

**Results:** Light Box Defecation Test (LBDT) showed EEG offspring presented less fecal pellets than CG offspring, ESG's more than CG's, and HG's less than ESG ( $p < 0.05$ ). Open-field Test (OFT) score of EEG was higher than CG offspring, of ESG's was lower than CG's, and HG's higher than ESG's. Irf7 and Ninj were screened, which were up-regulated in EEG, down-regulated in ESG ( $FC < 0.5$ ), and were neutralized in HG. Prenatal EE could positively promote the nervous system development, prenatal earthquake simulation could retard the nervous system development and Chinese herbal remedy (JKSQW) which could correct the retardation.

**Conclusion:** The negative-positive prenatal effect could contribute to altered gene expression of Irf7 and Ninj2 which also could play a key role in the improving function of JKSQW for the kidneys.

**Keywords:** Prenatal stress; Earthquake simulation; Light Box Defecation Test; Open-field Test; Irf7; Ninj2

**List of non-standard Abbreviation:** EE = environmental enrichment; EEG = environmental enrichment group; CG = control group; ESG = earthquake simulation group (ESG); HG = herbal group; LBDTL = light box defecation test; OFT = open field test'

## Introduction

Environmental enrichment (EE) is defined as an exposure of laboratory animals to physical and/or social stimulation superior to standard housing conditions (Halperin and Healey, 2011) [1]. EE gets more and more popular in the practice and study of animal husbandry, which has been proven to positively affect the physiological and psychological processes in animals (Toth et al., 2011). In neuroscience, EE shows benefits in neurodegenerative and psychiatric disorders, including sleep disorders, depression, anxiety, drug addiction, Alzheimer's and Parkinson's disease, and traumatic brain injury (Laviola et al., 2008). As a prenatal stress, earthquake simulation can significantly impact the psychological and intellectual development of fetus and birth outcomes (Zhang et al., 2008; Zhang et al., 2012). Pregnant female is extremely fragile in the face of earthquake. Physical and psychological damages are left behind to the mother and child (King et al., 2000). Oyarzo et al (Oyarzo et al., 2012) reported disasters such as earthquakes are associated with adverse perinatal outcomes that negatively impact the entire maternal-neonatal healthcare system. Tan et al (Tan et al., 2009) found that rates of birth defects after an earthquake were significantly higher than those pre-earthquake, whose spectrum was dramatically altered after earthquake, with the markedly increased occurrences of ear malformations; meanwhile the ratio of preterm birth post-earthquake was significantly increased than that of pre-earthquake.

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Prenatal effect plays an important role in the development of offspring in rats (Champagne et al., 2003; Tang et al., 2012). Due to a long term of perinatal mother-infant interaction in mammals, the growth and development of offspring are likely to be impacted by maternal influences, with psychological and physiological health left as long-term consequences (Simpson and Kelly, 2011). Convincingly, prenatal stress has been demonstrated able to affect pregnancy outcome and lead to early programming of brain functions leaving permanent changes in neuroendocrine regulation, gene expression and behavior of offspring in animal experiments (Gao et al., 2011; Belay et al., 2011; Gardner et al., 2009).

Prenatal stress from a simulation of earthquake is considered as a factor which impairs the kidney Qi (*shen qi*) (Leung et al., 2012), which is the fundamental root of the congenital constitution, controlling reproduction and development and holding orifice of labor, whence agility and emanates. Jin Kui Shen Qi Wan (JKSQW) is a representative herbal formula that strengthens kidney Qi, which restores the physiological functions of the kidney (Kolasani et al., 2011). Our previous studies found that maternal EE promoted the body weight and body length and behavior tests performance of offspring, and maternal earthquake retarded the body weight and body length and behavior tests performance. Meanwhile the retardation could be corrected by JKSQW (Zhang et al., 2012). Although many studies have focused on positive effect of the environmental enrichment and negative effect prenatal stress respectively, the trans-generation influence from positive-negative influences resulting from the maternal EE and prenatal stress, which induces development and genetic changes in offspring remains unknown (Rando and Verstrepen, 2007; Carone et al., 2010; Lazarov., et al., 2005).

Based on above information, we hypothesized certain genes was involved in the positive-negative intervention i.e. EE, earthquake simulation, and JKSQW treatment as underlying mechanism.

## Materials and Methods

### Grouping

Sixty-two Sprague-Dawley (SD) female rats (230g~270g) and 62 male rats (225g~261g) were involved in this research. The rats were housed in a room with a temperature of 22°C, 12 hour light/dark cycle and fed with food and water *ad libitum*. After a week of adaptation housing, the female rats were mated with the male rats. Pregnancy was confirmed with vaginal plug test. Then the 46 pregnant rats were randomized into four groups, EE group (EEG) (n=12), control group (CG) (n=11), earthquake simulation with conventional chow group (ESG) (n=11), and earthquake plus herbal group (HG) (n=12), and were housed under pregnant rat cages until the delivery. All the groups were transferred with equivalent stress during pregnancy. There was no statistical difference of gestation time detected or body weight of the first day after gestation (EEG: 235.21±1.59 (g), CG:234.87±2.20 (g), ESG: 234.98±1.95 (g) and HG: 35.16±1.96 (g), NOVA test,  $p>0.05$ ) in the four groups. After delivery, all the litters of both groups were housed with their mothers until the 25th day after birth.

### Housing

CG consisted of a standard pregnant rat rearing cage, 5-6 rats per cage. EEG consisted of a larger cage (100cm×100cm×100cm), all the 12 rats in the cage, with different objects (shelter, ladder, ball, stair, and tunnel), chews (nuts, toys), nesting material, and fetus education music twice a day in the morning and night. ESG and HG pregnant rats was housed in standard pregnant rat rearing cage as same as CG.

### Earthquake Simulation

The ESG cages housing pregnant rats were manually shaken up-and-down 3 times to simulate an initial earthquake and then were shaken for 50 times over the next 15 minutes to modulate an aftershock (Zhang et al., 2008; Zhang et al., 2012). The earthquake simulation was performed twice a day until delivery. Severity of the earthquake-shake was measured with a seismometer (DX-6Y2, Cheng Du Mei Huan Tech. Co. Ltd.), showing 9.6~10.5 of seismic intensity, 950 mg~1050 mg of vertical peak ground accelerations (PGA), which was similar to the PGA (1080 mg) of Wenchuan earthquake, May12, 2008, China.

**Chinese Herbal Formula Feed**

The feed of HG rats was supplemented with herbal medicine until delivery, which consisted of *Jin Kui Shen Qi Wan* (*Radix Aconiti Lateralis Preparata* 4.5g (Zhi Fu Zi), *Cortex Ramulus Cinnamomi* 9g (Rou Gui), *Radix Rehmanniae Preparata* 108g (Di Huang), *Fructus Corni Officinalis* 27g (Shan Zhu Yu), *Cortex Moutan Radicis* 27g (Mu Dan Pi), *Rhizoma Dioscoreae Oppositae* 27g (Shan Yao), *Sclerotium Poriae Cocos* 78g (Fu Ling), and *Rhizoma Alismatis Orientalis* 27g (Ze Xie)). According to the introduction from *Chinese Pharmacopoeia* (2005 Edition) and the produce process of the commercial pill of JKSQW, above eight herbs were grinded into fine powder respectively, were filtered and mixed together. Honey (35-50g per 100g herbal powder) and moderate water are added and stirred with the powders. The pill of the formula is molded with a pill machine in a weight of 0.2g per pill. Then the pill is desiccated. Then JKSQW pill was grinded and added to the conventional feed 0.5~0.6g/d per rat.

**Behavior Tests****Light Box Defecation Test (LBDT)**

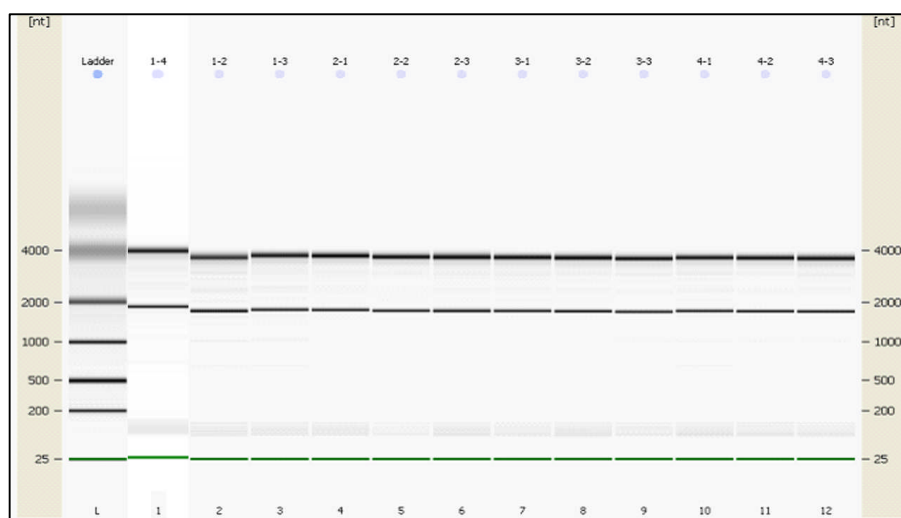
The test was performed 25 days after delivery in a dry and clean white plastic box (30cm×20cm), with an incandescent lamp hung 15 cm over the box. Immediately after a litter was put in the box, the incandescent lamp was turned on, resulting in brightness in the box. Fecal pellets were counted in two minutes (Barker et al., 2010).

**Open Field Test (OFT)**

A square board (90cm×90cm) was painted with yellow and white squares (15cm×15cm). The offspring of 25 days old was placed in the center of the board. We counted how many squares the offspring had crawled across in two minutes. One score was given only when the four paws of an offspring were in one square (Zhang et al., 2008).

**Gene Expression Profile Chip Experiments****RNA extraction and purification**

Total RNA was extracted using TRIZOL Reagent (Cat #15596-018, Life technologies, Carlsbad, CA, US) following the manufacturer's instructions and checked for a RIN number to inspect RNA integration by an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, US). Qualified total RNA was further purified by RNeasy mini kit (Cat#74106, QIAGEN, GmBH, Germany) and RNeasy micro kit (Cat#74004, QIAGEN, GmBH, Germany) and RNase-Free DNase Set (Cat#79254, QIAGEN, GmBH, Germany). (Figure 1).



**Figure 1:** Electrophoresis map of the RNAs of 12 samples. 1-2,1-3, and 1-4 refer to EEG, 2-1,2-3, and 2-3 refer to ESG, 3-1,3-2, and 3-3 refer to HG, 4-2,4-3, and 4-3 refer to CG. Two bands were clearly presented, showing high quality of RNA extraction and purification.

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## RNA amplification and labeling

Total RNA was amplified and labeled by Low Input Quick Amp Labeling Kit, One-Color (Cat # 5190-2305, Agilent technologies, Santa Clara, CA, US), following the manufacturer's instructions. Labeled cRNA were purified by RNeasy mini kit (Cat#74106, QIAGEN, GmbH, Germany).

## Hybridization

Each slide was hybridized with 1.65 µg Cy3-labeled cRNA using Gene Expression Hybridization Kit (Cat#5188-5242, Agilent technologies, Santa Clara, CA, US) in Hybridization Oven (Cat#G2545A, Agilent technologies, Santa Clara, CA, US), according to the manufacturer's instructions. After 17 hours hybridization, slides were washed in staining dishes (Cat#121, Thermo Shandon, Waltham, MA, US) with Gene Expression Wash Buffer Kit (Cat#5188-5327, Agilent technologies, Santa Clara, CA, US), following the manufacturer's instructions.

## Data Acquisition

Slides were scanned by Agilent Microarray Scanner (Cat#G2565CA, Agilent technologies, Santa Clara, CA, US) with default settings: dye channel: Green, Scan resolution = 5µm, PMT 100%, 10% , 16bit. Feature Extraction software 10.7 (Agilent technologies, Santa Clara, CA, US) Raw data were normalized by Quantile algorithm, Gene Spring Software 11.0 (Agilent technologies, Santa Clara, CA, US).

## Real-time PCR

Primers of Irf7 (Forward Primer: TGGCAGATGGAAGCTACC, Reverse Primer: GGCTATACAGGAACACGC, Product Length=154) and Ninj2 (Forward Primer: CCACCACCTTGGTCTTCATA, Reverse Primer: AGGCTGAAGTGGCTTTAG, Product Length=152) were designed with Primer Express 2.0 (Oebiotec, Shanghai, China). Reverse transcription was performed on PrimerScript RT reagent Kit (TaKaRa, DRR037A, Takara Biotechnology (Dalian) Co., Ltd. China). Total RNA (0.5 µg) was denatured at room temperature then mixed with the reagent in a final volume of 10 µl containing 50 µM oligo dT, 100 µM random primer, 0.5 mM dNTP and the manufacturer's buffer and Enzyme Mix. The RT reaction was conducted for 15 min at 37 °C, and 85 °C for 5s in ABI 9700. First-strand cDNA product was diluted in 100 µl distilled water in preparation for real-time PCR. qPCR was performed using Super Real PreMix (SYBR Green) kit (TIANGEN, FP204, Tiangen Biotech (Beijing) Co., Ltd. Beijing, China). Briefly, 1 µl of diluted cDNA product was used for 40-cycle three-step PCR in a Roche HOLD CYCLE Light Cycler 480 II.

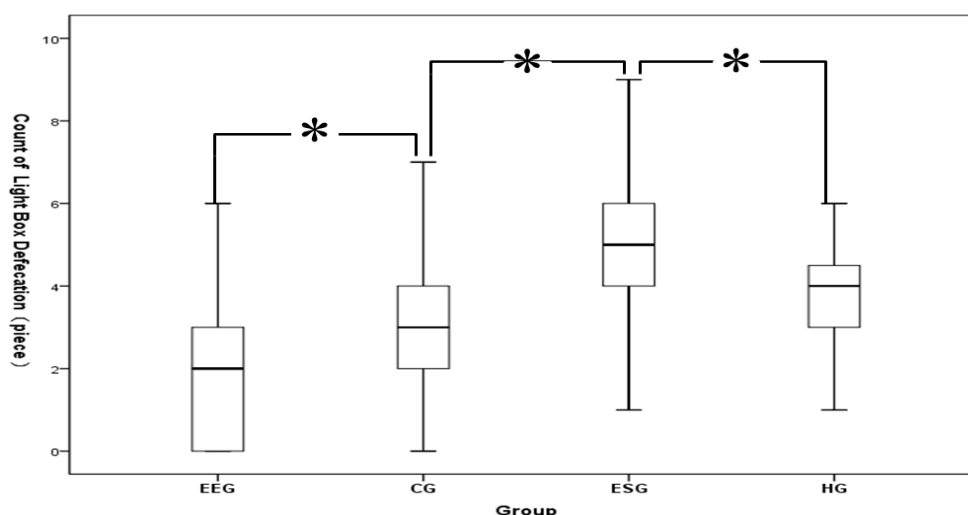
## Statistical Analysis

The development and behavioral tests were analyzed using a SPRENATAL STRESSSS (Statistical Package for the Social Sciences) version 19.0. A non-parametric test, Kruskal-Wallis test was performed to analyze group differences in LBBDT and OFT.

## Results

### Light Box Defecation Test

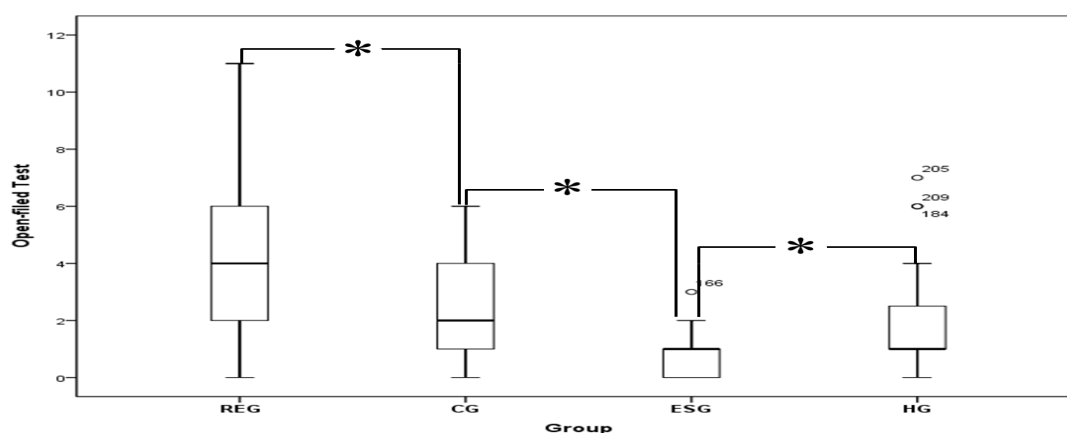
A Kruskal-Wallis test showed significant difference among the four group prenatal stress. Respectively, EEG offspring showed less fecal pellets than CG's (Mann-Whitney U=1055.50, Wilcoxon W=4295.50, Z=-3.894, p=0.00). ESG showed more fecal pellets than CG (Mann-Whitney U=323.00, Wilcoxon W=1358.00, Z=-2.977, p=0.00). HG showed less fecal pellets than ESG (Mann-Whitney U=249.50, Wilcoxon W=777.50, Z=-2.465, p=0.01). There was no statistical difference between CG and HG (Mann-Whitney U=637.50, Wilcoxon W=1672.50, Z=-0.87, p=0.38). (Figure 2)



**Figure 2:** Box plot of Light Box Defecation Test in the comparisons among EEG and CG, ESG, and HG: EEG showed fewer score than CG ( $p=0.00$ ), ESG showed more scores than CG ( $p=0.00$ ), and HG showed less scores than ESG ( $p=0.01$ ).

### Open-field Test

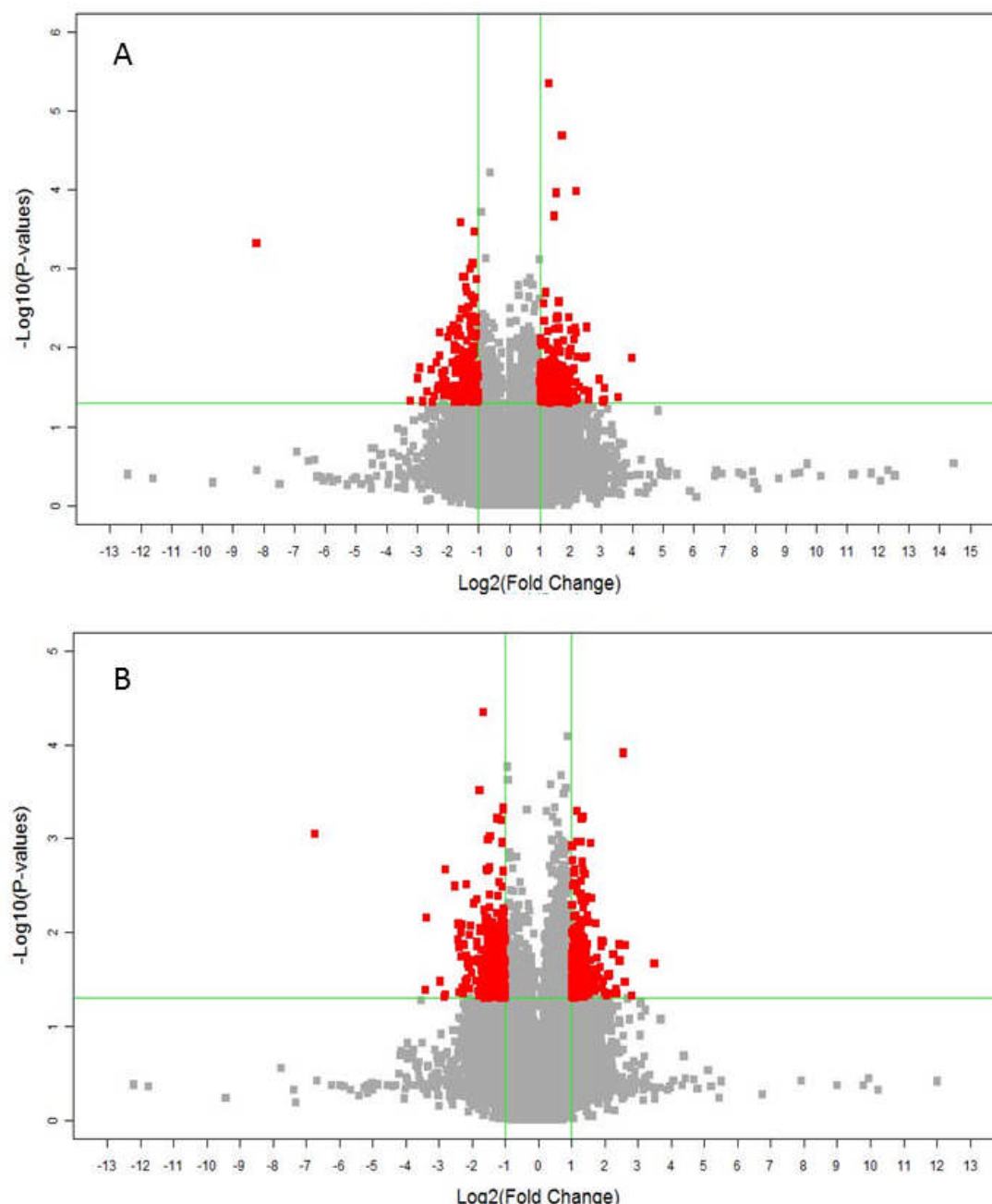
A Kruskal-Wallis test showed significant differences among the four group renal stress. Respectively, EEG showed more scores than CG (Mann-Whitney  $U=1448.500$ , Wilcoxon  $W=2529.50$ ,  $Z=-3.82$ ,  $p=0.00$ ); ESG showed less scores than CG (Mann-Whitney  $U=240.50$ , Wilcoxon  $W=565.50$ ,  $Z=-4.13$ ,  $p=0.00$ ); HG showed more scores than ESG (Mann-Whitney  $U=275.500$ , Wilcoxon  $W=600.500$ ,  $Z=-2.543$ ,  $p=0.01$ ) (Figure 3)



**Figure 3:** Box plot of Open-field Test in the comparisons among EEG and CG, ESG, and HG: EEG showed less scores than CG ( $p=0.00$ ), ESG showed more scores than CG ( $p=0.00$ ), and HG showed less scores than ESG ( $p=0.01$ ).

### Genes simultaneously differently expressed in EEG vs CG, ESG vs CG, HG vs CG (no change)

EEG and CG groups differed significantly in gene expression profile: 25 genes were up-regulated and 23 genes were down-regulated (Figure 4\_A). Gene expression profile showed 81 genes up-regulated and 39 genes down-regulated ((Figure 4\_B) in ESG vs CG comparison. Among the 120 differently expressed genes in ESG vs CG, the difference of 85 genes disappeared in HG vs CG (no change) ( $p>0.05$ ), indicating JKSQW could alter the 85 genes. Interestingly, Irf 7 and Ninj2 were found differently expressed in the three comparisons (Figure3): Comparing with CG, Irf7 and Ninj2 up-regulated in EEG ( $FC>2$ ), down-regulated in ESG ( $FC<0.5$ ), and whose expression was neutralized in HG ( $0.05<FC<2$ ) (Figure 5).

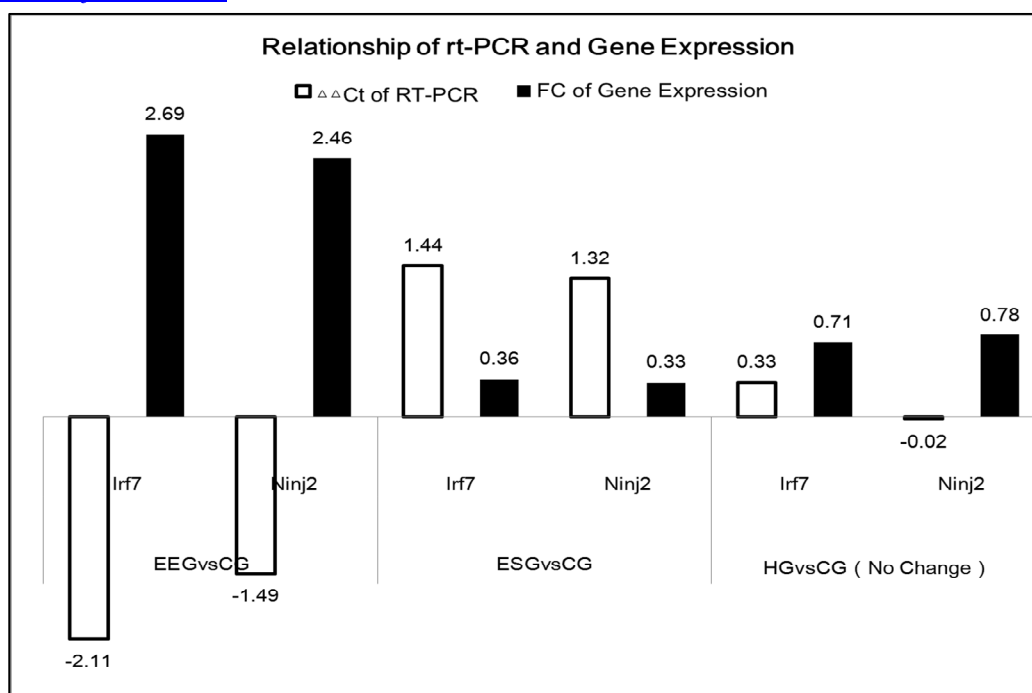


**Figure 4:** Volcano plots differentially expressed genes in EEGvsCG and ESG vs CG. Red areas refer to  $p < 0.05$  and folder change  $\geq 2$ . A: EEG vs CG, 25 genes were up-regulated and 23 genes were down-regulated. B: ESG vs CG 81 genes were up-regulated and 39 genes were down-regulated.

## Discussion

Substantial evidences from animal researches indicate that EE can positively influence the behavioral development of offspring and PRENATAL STRESS negatively affect that. (Kofman, 2002; Mychasiuk et al., 2012). EE and PRENATAL STRESS have been shown to cause significant changes in the expression of genes whose products are involved in neuronal structure, plasticity, and neurotransmission, but the genetic changes of the offspring resulting from the prenatal inventions remain unclear (Meaney, 2001; Fumagalli et al., 2005). Findings of this study showed EEG offspring defecated less fecal pellets than CG's, ESG defecated more fecal pellets than CG's, and HG reduced the over-defecation of ESG offspring.

Defecation is taken to reflect emotionality, and high defecation score is correlated to more anxiety in rats (van der Staay et al., 2009; Ramos et al., 2008). Cherian et al. (2009) reported offspring with prenatal stress (both male and female) showed significantly more fecal pellets compared to respective control offspring. Few researches are available on how maternal EE influences offspring's defecation and controversy still exists. Peña et al found there was no statistical difference of defecation scores between EE and control



**Figure 5:** RT-PCR validations of the *Irf7* and *Ninj2* from gene expression profile chip prenatal stress.  $\Delta\Delta C_t < 0$  indicates the target genes were hyper-expressed in the three comparisons while  $\Delta\Delta C_t > 0$  indicates the target genes were hypo-expressed. Folder change  $> 2$  indicates the target genes were hyper-expressed in the three comparisons while Folder change  $< 0.5$  indicates the target genes were hypo-expressed.

(Peña et al., 2009), similar result was reported by Stewart et al (Stewart et al., 2012). However our study found that EEG showed lower defecation score than CG. Previous researchers found that with prenatally stressed offspring showing more defecation than that of control group (Buitelaar et al., 2003). But controversy still exists. Shachar-Dadon et al reported that prenatal preconceptional stress (PCS) influenced affective and social behavior in offspring, excluding defecation performance (Shachar-Dadon et al., 2009). Our study found that EEG showed more defecation score than CG, and JKSQW reduced the increased defecation score. Thus, prenatal EEG could reduce the anxiety behavior in offspring and prenatal earthquake simulation enhances the anxiety behavior in offspring. Interestingly, JKSQW in HG could neutralize the increased anxiety performance. In Chinese medicine, kidney function is disturbed by the prenatal stress like earthquake and restored by JKSQW. In OFT, it was demonstrated that prenatal EE could improve exploration performance. On the contrary, prenatal earthquake simulation reduced OFT performance. The reduction could be recovered by JKSQW. JKSQW is a representative herbal formula to tonify kidney qi, which restores the physiological functions of kidney. In summary, prenatal EE significantly promoted the locomotor activity in offspring, indicating that maternal enrichment benefited postnatal behaviors e.g. OFT performance in offspring. Meanwhile, prenatal EE reduced offspring's anxiety. On the contrary, prenatal stress (earthquake simulation mentioned above) increased offspring's anxiety and retarded postnatal behaviors test performance. Altered behavior and emotionality traits were recovered by JKSQW, supporting the effectiveness of Chinese herb remedy in rodents in lab (Kolasani et al., 2011).

At present, *Irf7* (interferon (IFN) regulatory factor 7) is believed to be involved in multiple immunology process (Erickson and Gale, 2008; Colina et al., 2008). We found that *Irf7* up-expressed in EEG offspring, down-expressed in ESG offspring, and normally expressed in HG. It is reported that all elements of IFN responses, whether the systemic production of IFN in innate immunity or the local action of IFN from plasmacytoid dendritic cells in adaptive immunity, are under the control of *Irf7* (Honda et al., 2005). Hannah et al (Hannah et al., 2008) reported that induction of pattern recognition receptors (PRRs; Tlr7 and Rlg-I), expression of antiviral genes (Myd88, Visa, Jun, *Irf7*, *Ifn $\beta$* , *Ifnar1*, *Jak2*, *Stat3*, and *Mx2*), and production of *Mx* protein was elevated in the lungs of intact females compared with intact males (Araki and Milbrandt, 2000). Previous studies revealed that prenatal stress produced alterations in immune function that can be reversed by enriched environment and Chinese medicine (Sun et al., 2010). This finding demonstrated that positive and negative prenatal experiences could significantly alter offspring developmental and immunity trajectories, JKSQW effects too, which might provide a clinical orientation, i.e. prenatal intervention for prenatal developmental and immunological disorders. To our knowledge, no connection between *Irf7* and behavioral development has been established in current reports. Lukasz et al (Lukasz et al., 2012) reported that augmented interferon signaling in hippocampus might represent a common molecular imprint of environmental insults (social isolation) associated with neuropsychiatric illnesses like schizophrenia. Therefore it's



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reasonable to deduce that prenatal stress might leave molecular imprint in hippocampus through interferon signaling pathways, which leads to behavior and emotionality alteration in offspring. Concerning this, further study should be carried out.

Ninjurin2 (Ninj2), firstly reported in 2000, is a homolog of a homophilic cellular adhesion molecule, which mediates cell-to-cell and cell-to-extracellular matrix interaction during development, differentiation and regeneration of nervous system (Araki and Milbrandt, 2000; Lin et al., 2011). Ninj2 was also reported to be a vascular susceptibility gene and associated with Alzheimer's disease risk and ischemic stroke (Lin et al., 2011; Wan et al., 2011) and vascular dementia (Iemolo et al., 2009). To our knowledge, it is the first time involving Ninj2 in negative and positive prenatal interventions. Our study found that ninj2 up-expressed in EEG offspring, down-expressed in ESG offspring, and normally expressed in HG, indicating, Ninj2 might play a role in the genetic influences from prenatal positive and negative experiences, and further study is required.

## Conclusion

The findings of this study demonstrate prenatal EE could positively promote the nervous system development, earthquake stress could retard the nervous system development and herbal formula JKSQW could rectify the retardation. The negative-positive prenatal effect could result from changed expression of Irf7 and Ninj2. Meanwhile Irf7 and Ninj2 could play a key role in improving function of JKSQW.

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**Conflict of interest statement:** No conflict of interest declared.

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