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Effects of Spirulina on GABA Receptor Gene Expression in Salicylate-Induced Tinnitus

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Abstract

Objectives: The function of γ-amino butyric acid receptor (GR) was related with tinnitus. But, the effects of *Spirulina platensis* water extract (SP) on the mRNA expression of *GRAβ3* in mice with tinnitus were still unclear.

Method: Eighteen SAMP8 mice were divided into the control group (intraperitoneal injection of saline, once per day), the tinnitus group (intraperitoneal injection of salicylate, 300 mg/kg body weight once per day), and the spirulina group [intraperitoneal injection of salicylate, 300 mg/kg body weight and oral SP supplementation (1000 mg/kg body weight) once per day]. Effects of SP on the mRNA expression of *GRAβ3* in the cochlea and brain of mice were studied for 4 days.

Results: Compared to the control group, the tinnitus group had significantly higher tinnitus scores and lower mRNA expression of *GRAβ3* gene in the cochlear, brainstem, hippocampus and parahippocampus, temporal lobes, and the frontal lobes. On the other hand, the spirulina group had significantly lower tinnitus scores and higher *GRAβ3* gene expression than the tinnitus group in all tested areas.

Conclusion: SP could reduce salicylate-induced tinnitus possibly *via* increasing the salicylate-induced down-regulation of *GRAβ3* gene expression.

Keywords: spirulina, tinnitus, salicylate, γ-amino butyric acid receptor gene.

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INTRODUCTION

Tinnitus is a common complex disease and often results from auditory dysfunction, although many other etiologies were reported[1]. Some mechanisms including the neural receptor theory were proposed in these decades. For example, gene expression and/or function of N-methyl D-aspartate (NMDA) receptor (NR) increased in the inner ear and/or auditory nervous system[2,3]. On the contrary, function and/or gene expression of the γ-amino butyric acid (GABA) receptor (GR) and cannabinoid receptor in some brain areas decreased in the condition of tinnitus[4-6]. In addition, oxidative stress damage and/or neural inflammation in the peripheral and central auditory system were also important mechanisms of tinnitus[7-10].

Some literatures in traditional Chinese medicine had said that algae could prevent hearing loss and/or tinnitus. Previously, our study group had found that *Spirulina platensis* water extract (SP) and/or its active component [c-phycocyanin (CPC)] could decrease tinnitus, and the mRNA expression of *NR2B* and pro-inflammatory genes in the cochlea and brain during salicylate-induced tinnitus[8]. SP could modulate the endogenous antioxidant gene expression and reduce malondialdehyde levels in some brain areas[9]. Also, we found that SP could prevent salicylate-induced up-regulation of Na+-K+-2Cl-co-trans *-porter 2 (KCC2*) gene in the cochlear and brain[10].

However, effects of SP on the mRNA expression of GR gene in tinnitus were still unknown. In this study, we aimed to investigate the effects of SP on the salicylate-induced tinnitus and the mRNA level of *GRAβ3* gene in the cochlea and tinnitus-related brain areas.

METHODOLOGY

Animals and Groups

Eighteen male SAMP8 mice of 3 months old were divided into three groups: the control group (saline injection per day for 4 days), the tinnitus group (salicylate injection, 300 mg/kg body weight per day for 4 days), and the spirulina group (salicylate injection, 300 mg/kg body weight and SP treatment, 1000 mg/kg body weight per day for 4 days). Institutional Animal Care and Use Committee of Dalin Tzu Chi Hospital had approved the protocol (0981117-03) used in this study.

The component of SP had 35%-45% polysaccharides, 15%-25% phycobiliproteins, 10%-20% proteins, 10%-12% ash, and 5%-8% water (Far East Bio-Tec Co., Ltd., Taipei, Taiwan).

Active avoidance task

All mice were conditioned to perform an active avoidance task in a box, which included a safe area and an electrical floor[11].

The protocol of this task had 6 sessions (60 trials totally) and performed once a day for 5 days. A 10 kHz pure tone of 50 dB sound pressure level (SPL) with 3 seconds duration (conditioning stimulus), and an electric foot-shock of 3.7 mA for 30 seconds (unconditioned stimulus) were given in each trial. The mice ran into the safe area after they received these two stimuli. Only conditioned mice, which succeeded the task at least 80% in three consecutive sessions, were selected into subsequent experiments.

Testing of tinnitus

An active avoidance task of 10 trials was performed 2 hours after intra-peritoneal saline injection (the control group), intraperitoneal sodium salicylate injection (300 mg/kg body weight) (the tinnitus group), or intra-peritoneal sodium salicylate injection (300 mg/kg body weight) plus oral SP supplementation (1000 mg/kg body weight) (the spirulina group) for 4 days. During tinnitus testing, a 10 kHz pure tone of 70 dBSPL with 3 seconds duration was given first for 3 seconds in each trial. We observed the mice movement for 5 seconds to see whether they ran into the safe area or not. If so, the mice were taken back to the electric floor for the ongoing observation. If not, electrical shock was given to remind the mice to ran into the safe area. Then, the mice were taken back on the electric floor for ongoing observation. After the above two conditions, we observed the total numbers of times the mice ran into the safe area during the inter-trial period of 1 minute of 10 trials as the tinnitus score.

RNA extraction

The pairs of cochlea and brain tissue were immediately taken and stored in -80°C refrigerator. RNA isolation was done using the RNA-Bee isolation reagent (Friendswood, USA) with a tissue homogenizer. The RNA quality was checked by Agilent Bioanalyzer 2100 and the ratio of absorbance measurements at 260 and 280 nm using the nanodrop.

Reverse transcription-polymerase chain reaction (RT-PCR)

The cDNA was transformed from total RNA by reverse transcription using MMLV high performance reverse transcriptase (Epicentre Biotechnologies, USA) in a P × 2 Thermal cycler (Thermo Electron Corporation Bioscience Technologies Division, USA). For each RT reaction, a positive control was performed with 1 μg of total RNA. RT was carried out at 37°C for 1 hour. For PCR amplification, 3 μL cDNA and primers were used with DreamTaqTM DNA polymerase (Fermentase, USA). The primers were: *GRAβ3* -F, 5'-GCCATCGACATGTACCTGA -3', *GRAβ3*-R, 5'-GAATTCCTGGTGTACCCAA -3'; *GAPDH*-F, 5'- ATGACATCAAGAAGGTGGTG-3', and *GAPDH* -R,5'-CATACC

AGGAAATGAGCTTG-3' (Protech-Taiwan, Taiwan). The thermal cycling conditions for PCR were adjusted to the following: 3 minutes initial set-up at 95°C; followed by 50 cycles, each of which consisted of 45 seconds of denaturation at 95°C, 45 seconds of annealing at 46°C, and 45 seconds of extension at 72°C for *GRAβ3* gene;

of 45 seconds of denaturation at 95°C, 45 seconds of annealing at 50°C, and 45 seconds of extension at 72°C for *GAPDH* gene. A final 10 minutes at 72°C for both genes.

Quantitation of PCR products

The cDNA products were measured by the Mini Horizontal Electrophoresis System (MJ-105/ MP-100, Major Science, Taiwan) and by E-Box-1000/26M Inspection Certificate and Analysis Systems (E-Box Spp-010 E-capt software, USA). The expression level of *GRAβ3* gene was presented as relative ratios in comparison to GAPDH.

Statistical analysis

The tinnitus scores were compared between three groups by one-way analysis of variance (ANOVA) with post-hoc Bonferroni correction. And, the mRNA expression levels of *GRAβ3* gene at each site were compared separately between three study groups by one-way ANOVA with post-hoc Bonferroni correction. All above analysis were performed by the commercialized software "STATA10", and p values <0.05 were considered statistically significant.

RESULTS

Figure 1 showed the tinnitus scores of all groups before and 4 days after salicylate injection. The tinnitus scores were not significantly different in three groups before intra-peritoneal salicylate injection (mean ± standard deviation=1.0 ± 2.4, 0.0 ± 0.0, 0.5 ± 1.2, respectively; one-way ANOVA, p=0.5615). Compared to the control group (1.8 ± 4.5), the tinnitus group (10.7 ± 6.7), but not the spirulina group (3.2 ± 1.5 had significantly higher scores on the day 4 after salicylate injection (one-way ANOVA, p=0.0113; post-hoc Bonferroni analysis, p=0.016 and 1.000, respectively). And, the spirulina group had significantly lower scores than the tinnitus group on the day 4 after salicylate injection (post-hoc Bonferroni analysis, p=0.044). Figures 2-6 showed the

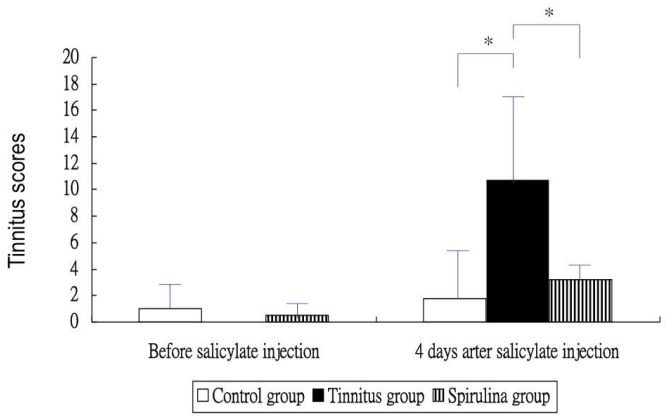

Figure 1. The tinnitus scores of all groups before and 4 days after salicylate injection. The tinnitus scores were not significantly different in three groups before intra-peritoneal salicylate injection (p=0.8921). Compared to the control group, the tinnitus group, but not the spirulina group, had significantly higher scores after salicylate injection. And, the spirulina group had significantly lower scores than the tinnitus group after salicylate injection (*<0.05).

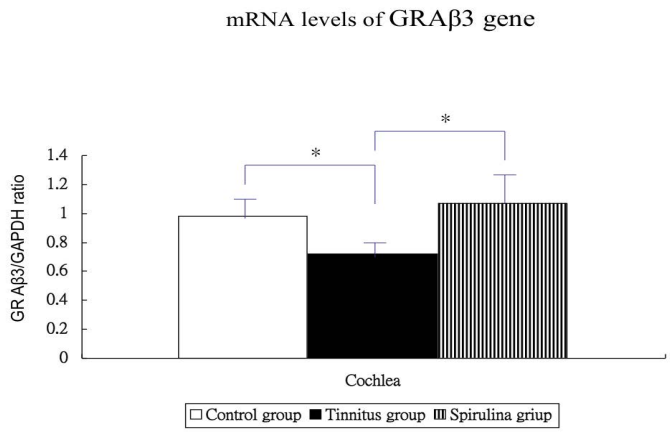

Figure 2. The mRNA expression levels of the *GRA®3* gene in the cochlea. The tinnitus group had significantly lower mRNA expression of *GRA®3* gene than the control group. And, the spirulina group had significantly higher mRNA expression of *GRA®3* gene than the tinnitus group (*<0.05).

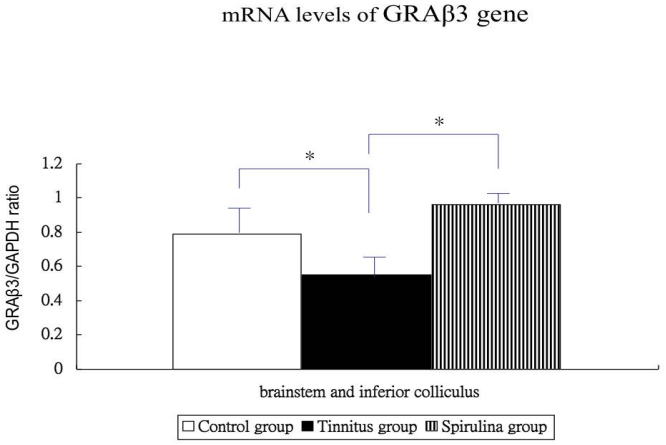

Figure 3. The mRNA expression levels of the *GRA®3* gene in the brainstem and IC. The tinnitus group had significantly lower mRNA expression of *GRA®3* gene than the control group. And, the spirulina group had significantly higher mRNA expression of *GRA®3* gene than the tinnitus group (*<0.05).

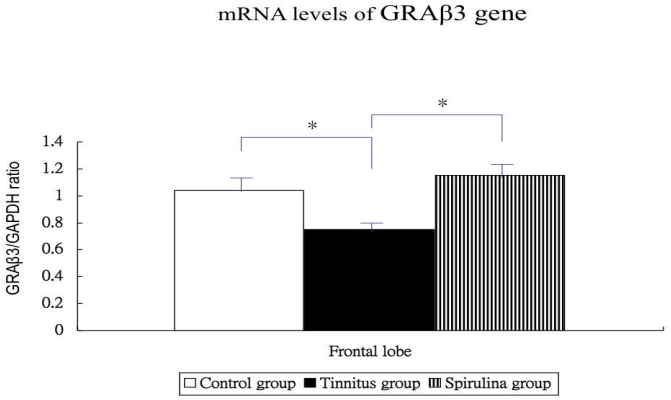

Figure 4. The mRNA expression levels of the *GRA®3* gene in the hippocampus and para-hippocampus. The tinnitus group had significantly lower mRNA expression of *GRA®3* gene than the control group. And, the spirulina group had significantly higher mRNA expression of *GRA®3* gene than the tinnitus group (*<0.05).

mRNA expression levels of the *GRAβ3* gene in different sites of three groups. The tinnitus group had significantly lower mRNA expression of *GRAβ3* gene than the control group in the cochlea (0.72 ± 0.06 versus 0.98 ± 0.12; one-way ANOVA, p=0.0026; post-hoc Bonferroni

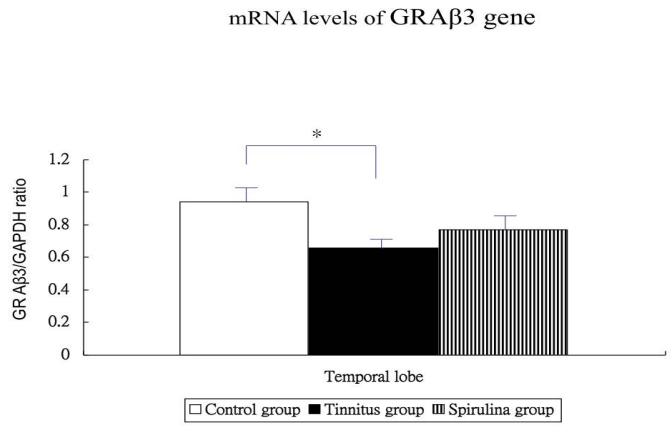

Figure 5. The mRNA expression levels of the *GRA®3* gene in the temporal lobes. The tinnitus group had significantly lower mRNA expression of *GRA®3* gene than the control group. And, the spirulina group had higher of borderline significance mRNA expression of *GRA®3* gene than the tinnitus group (*<0.05).

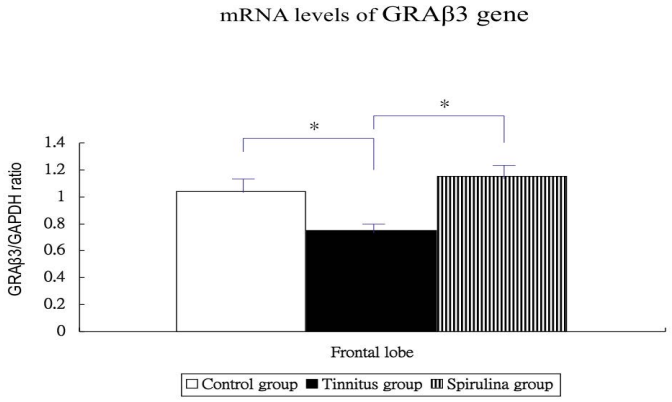

Figure 6. The mRNA expression levels of the *GRA®3* gene in the frontal lobes. The tinnitus group had significantly lower mRNA expression of *GRA®3* gene than the control group. And, the spirulina group had significantly higher mRNA expression of *GRA®3* gene than the tinnitus group (*<0.05).

analysis, p=0.023) (Figure 2), in the brainstem and IC [0.55 ± 0.12 versus 0.79 ± 0.18; one-way ANOVA, p=0.0003; post-hoc Bonferroni analysis, p=0.018] (Figure 3), in the temporal lobes [0.66 ± 0.04 versus 0.94 ± 0.09; one-way ANOVA, p<0.0001; post-hoc Bonferroni analysis, p<0.001] (Figure 5) , in the frontal lobes [0.75 ± 0.08 versus 1.04 ± 0.11; one-way ANOVA, p<0.0001; post-hoc Bonferroni analysis, p<0.001] (Figure 6), and in the hippocampus and parahippocampus [0.83 ± 0.07 versus 1.03 ± 0.11; one-way ANOVA, p=0.0012; post-hoc Bonferroni analysis, p=0.021] (Figure 4). The spirulina group had significantly higher mRNA expression of *GRAβ3* gene than the tinnitus group in the cochlea (1.07 ± 0.22, post-hoc Bonferroni analysis, p=0.003) (Figure 2), in the brainstem and IC (0.96 ± 0.08, post-hoc Bonferroni analysis, p<0.001) (Figure 3), in the temporal lobes (0.77±0.08, post-hoc Bonferroni analysis, p=0.050) (Figure 5), in the frontal lobes (1.15 ± 0.10, post-hoc Bonferroni analysis, p<0.001) (Figure 6), and in the hippocampus and para-hippocampus (1.12 ± 0.14, post-hoc Bonferroni analysis, p=0.001) (Figure 4).

DISCUSSION

This experimental study showed that mRNA expression of *GRAβ3* gene decreased in the cochlea and several tinnitus-related areas in the brain after high-dose salicylate injection. Meanwhile, SP could reduce salicylate-induced tinnitus, and prevent salicylate-induced down-regulation of *GRAβ3* gene in the cochlea and some tinnitus-related areas. GABA, an inhibitory neurotransmitter, exerted its effects through ionotropic GRA and *GRC*, and *GRB* in the brain. *GRA* is a ligand-gated chloride channel[12]. Phosphorylation of *GRAβ1* subunits reduced *GRA* currents, whereas phosphorylation of *GRAβ3* subunits increased *GRA* currents[13]. Salicylate increased the sound-evoked field potentials in the auditory cortex *via* interfering the GABA-mediated inhibition[14]. Conversely, activation of GR in IC and auditory cortex could reduce tinnitus in animals[4,15]. Currently, we showed that mRNA expression of *GRAβ3* gene decreased in the cochlea and several tinnitus-related brain areas during salicylate-induced tinnitus. Collectively, these data suggested that GR was negatively associated with tinnitus at the mRNA and protein levels along the peripheral and central auditory pathways.

Previous studies showed that SP could prevent neuro-inflammatory diseases by inhibiting nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and COX[16], and modulating the activities and/or mRNA expressions of endogeneous antioxidant genes[17]. Although accumulation of arachidonic acid (AA) caused by inhibition of COX could potentiate NR currents in salicylate-induced tinnitus[18], our previous and current studies have suggested that anti-inflammation was the dominant mechanism underlying the beneficial effect of SP on salicylate-induced tinnitus.

Inflammatory and/or oxidative stress insults could violate the functions of cell membrane, cytoplasm and nucleus. For example, severities of inflammatory responses were correlated with GABA gene expression in post-status epilepticus[19]. The activities of *GRA* increased in inflammation-induced memory loss[20]. Activation of pro-inflammatory cytokines, *GRA* and *GRC* genes were found simultaneously in the brain in status epilepticus[21]. On the other hand, inflammatory stimuli could regulate alternative splicing of *GRB* gene in Alzheimer disease[22]. Therefore, it is reasonable to suppose that high dose salicylate might induce tinnitus *via* modulation mRNA expression of *GRAβ3* gene. Second, it is also reasonable to suppose that anti-inflammatory agent and/or antioxidants could reduce salicylate-induced tinnitus as we showed here.

CONCLUSION

The mRNA expression of *GRAβ3* gene decreased in the cochlea and several tinnitus-related areas in the brain during salicylate-induced tinnitus. And, SP, which exerts anti-inflammatory and anti-oxidative capacities, could reduce salicylate-induced tinnitus and prevent

salicylate-induced down-regulation of *GRAβ3* gene in the cochlea and some tinnitus-related areas.

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