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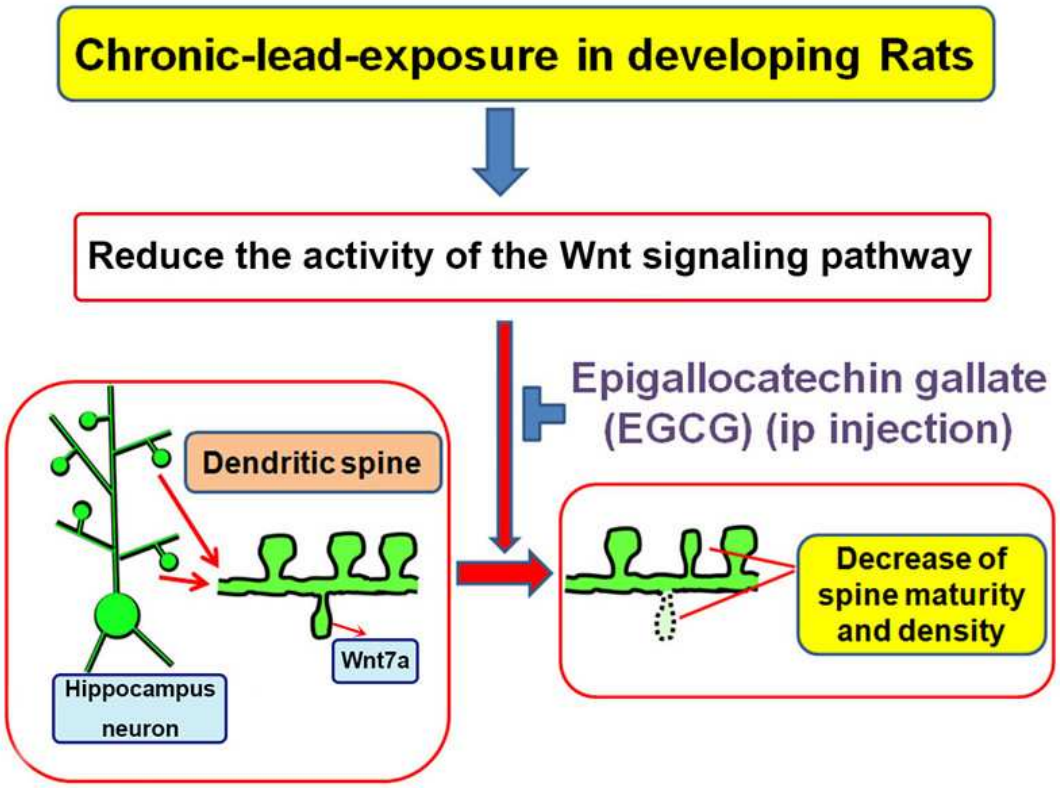
Graphical Abstract



Graphical abstract: Chronic lead exposure in the developing rats could decrease the spine formation and maturation in hippocampus. It may be involved down-regulate the Wnt7a expression and reduce Wnt/β-catenin signaling pathway activity. Epigallocatechin-3-gallate (EGCG), a compound of catechin polyphenols, could significantly reverse the lead-related spine damage by increasing Wnt/β-catenin signaling activity.

Wnt/β-catenin Signaling Involved in Protective Effect of EGCG on Lead-induced Impairments of Spine Formation in Rat Hippocampus

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ABSTRACT

Wnt/β-catenin signaling pathway has been implicated in development of dendritic spines, which is the structural basis for induction of long-term potentiation (LTP). Our previous study has demonstrated that developmental lead exposure causes spine damage on hippocampal pyramidal neurons by decreasing Wnt/β-catenin signaling pathway activity. Epigallocatechin-3-gallate (EGCG), the most abundant catechin in green tea, could recover impaired hippocampal-dependent LTP in lead-exposed rats. The present study is to investigate whether this EGCG protective function is by regulating Wnt/β-catenin signaling pathway to refine spine formation in lead-exposed rats. Sprague–Dawley rat pups were exposed to lead from parturition to weaning and EGCG (10, 25 and 50 mg/kg) was given intraperitoneally (ip) from postnatal day 14 (PND 14) to PND 21. We found that lead exposure significantly decreased dendritic spine density and spine head size of pyramidal neurons in hippocampal CA1 areas, and EGCG (10 and 25 mg/kg) reversed the lead-induced spine damage. In addition, EGCG (10 and 25 mg/kg) recovered the expression of Wnt7a and β-catenin phosphorylation after lead-exposure. However, 50 mg/kg of EGCG failed to restore the spine morphology and Wnt/β-catenin pathway activity on lead-exposed rats. Furthermore, in cultured hippocampal neurons, EGCG did not exert any protective effect on Pb2+-induced damages when Wnt7a shRNA applied. Our results demonstrate that EGCG (within a certain dose range) has a significant protective effect on the spine formation and maturation through Wnt/β-catenin signaling in lead-exposed young rats, which involves up-regulation of Wnt7a expression and attenuation of phospho-β-catenin expression. EGCG can be a potential complementary agent in the treatment of lead intoxication.

Keywords: Lead (Pb^{2+}); EGCG; Hippocampus; Spine; Wnt7a

1. INTRODUCTION

Green tea contains polyphenols known as catechins, which has been associated with health benefits[1]. The most abundant and potent catechin in green tea is (-)-Epigallocatechin-3-gallate (EGCG), which provides obvious protective effects to varieties of chronic pathological conditions, including diabetes, cancer, Parkinson's disease (PD), Alzheimer's disease (AD), stroke, and obesity[2-4]. It has been confirmed that EGCG could easily pass through the blood–brain barrier and then exert its protective effects on the central nervous system (CNS)[5-7]. In animal model of PD, EGCG effectively prevents the striatal dopamine depletion and substantia nigra dopaminergic neuronal loss[8]. In vitro, it attenuates L-DOPA-induced apoptosis in rat PC12 cells[9]. In AβPP/PS-1 double mutant transgenic mouse model of AD, EGCG reduces Alzheimer's amyloid-induced mitochondrial dysfunction in hippocampus, cortex, and striatum[10].

Lead (Pb^{2+}) is one of well-established environmental poisons, and its general toxic effects, particularly on children healthy, continue to be a major public health issue worldwide[11-14]. Developmental Pb^{2+} exposure has been considered as a high-risk factor for the attention deficit hyperactivity disorder (ADHD) in children[15, 16]. Additionally, Pb^{2+} can impair induction of hippocampal-dependent long-term potentiation (LTP, a form of synaptic plasticity) [13, 17]. Pb^{2+} poisoning may interfere with neurotransmitter release from presynaptic vesicle, as well as postsynaptic NMDAR expression and its downstream signaling[18-20]. It has been proved that

morphological alteration of spines, small protrusions in dendrite of spiny neurons, is associated with induction of LTP in hippocampus[21, 22]. Our previous study shows that there is an obvious decrease of dendritic spine formation after developmental Pb^{2+} exposure in rats[23], which provide directly structural evidence for Pb^{2+} induced synaptic plasticity impairment.

In view of EGCG neuroprotective properties, it is expected to mitigate Pb^{2+}-induced neurotoxicity. It has been shown that EGCG can reverse the decease of hippocampal LTP induction and maintenance in chronic Pb^{2+} exposed rats[24]. Although EGCG exerts protective function against synaptic plasticity deficits, there is no morphological evidence to support this phenotype. The Wnt/β-catenin signaling pathway has been reported to participate in the regulation of synapse formation and remodeling[25]. For example, Wnt7a (one of Wnt ligands) has been recently shown to promote dendritic spine growth and synaptic strength[26]. Our previous study demonstrates that down regulating of Wnt/β-catenin signaling activity involves in the synaptic damage caused by developmental Pb^{2+} exposure for decreasing of Wnt7a expression[23]. Whether the protection of EGCG on LTP induction in Pb^{2+} exposed rats is through the Wnt/β-catenin signaling pathway to regulate spine formation remains elusive.

In the present study, we attempted to investigate effects of EGCG on spine formation and maturation in rat hippocampus following chronic Pb^{2+} exposure. We also explored whether EGCG is dependent on regulating Wnt/β-catenin pathway to

protect spine morphology.

2. MATERIALS AND METHODS

2.1 Animals and experimental design

The Sprague–Dawley (SD) rats were obtained from the Laboratory Animal Center, Anhui Medical University, P. R. China and were maintained in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 2010). SD dams were fed with standard laboratory chow and distilled water and kept at a carefully controlled ambient temperature (20 ± 3 ℃) and relative humidity (50 ± 10%). On parturition day, the dams were randomly divided into five groups as follow: (1)control; (2)Pb^{2+}-exposed; (3)Pb^{2+} + EGCG 10 mg/kg; (4) Pb^{2+} + EGCG 25 mg/kg; (5)Pb^{2+} + EGCG 50 mg/kg.

The protocol for Pb^{2+} exposure had been described in our previous study[27]. The first day of birth was considered PND1. The Pb^{2+}-exposed and Pb^{2+} + EGCG pups acquired lead via milk of dams whose drinking water contained 0.05% Pb^{2+} acetate (250 ppm, 30 ml/day) from parturition to weaning (PND 21), while the control dams remained on distilled water throughout the lactation period. The pups were exposed to Pb^{2+} only via mother's milk. On PND 14 to 21, the offspring of three Pb^{2+} + EGCG groups were injected intraperitoneally (ip) daily with EGCG (10 mg/kg, 25 mg/kg and 50 mg/kg respectively), for this period has been considered as a key offstage for the nervous system development of rodent animals[28]. The EGCG was obtained from

Leshan Yujia Tea Science and Technology Development Co, Ltd, and dissolved in physiological saline[29, 30]. In the same period, the offspring of control and Pb^{2+} -exposed groups were injected daily with physiological saline. In all of the five groups, equal numbers of females and males were used. Only one pup per litter was selected for given experimental measure. After the last administration of EGCG (on PND 21), the animals were given 1 day of rest and then sacrificed under deeply anesthetized with CO_2. Half brain of each pup was emerged in the Golgi-Cox solution for neurons morphological staining, and the other half was collected for Pb^{2+} concentration determination and protein quantitative analysis.

2.2 Hippocampus tissue Pb^{2+} concentration estimation

The Pb^{2+} concentration in the hippocampus of pups was detected using Graphite furnace atomic absorption spectrometry method (GFAAS)[31]. Briefly, the hippocampus (<0.5 g) was added with nitric acid (excellent pure GR, 4 ml) and 30% hydrogen peroxide (AR, 2 ml) overnight, then digested for 30 min in the microwave nitrate pyrolysis furnace (EMR marsxpress certificate, VB 20). Then the Pb^{2+} concentration within sample was detected with Graphite furnace atomic absorption spectrometry (The PerkinElmer AAnalyst™ 800, USA).

2.3 Golgi-Cox Staining

To analyze the alteration of dendritic spine morphology, rat brains were processed with Golgi-Cox staining using the procedure described previously[32]. In brief, the

brains were firstly stored in the dark for two days (37 ^{o}C) in Golgi-Cox solution, and then were sectioned at a thickness of 200 μm in the coronal plane with a vibratome (VT1000S, Leica, Germany). All sections with hippocampus were collected and stained with ammonia for 60 min, followed by Kodak Film Fix for 20 min, and then dehydrated, cleared, and mounted using a resinous medium. At last, the pyramidal neurons in hippocampus CA1 were imaged with a Nikon wide field microscope (Eclipse 80i) by using a 40x objective. Next, Images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Neurons that had a pyramidal-shaped cell body and a clear secondary dendrite were chosen for analysis. Total dendritic protrusion density was counted according to previous method[33], and the spines density was estimated as the number of spines on each terminal dendrite per 10 μm. Furthermore, the head size of mushroom-like spines on pyramidal and granule neurons was measured by plugin for ImageJ software[34].

2.4 Western Blotting

The hippocampus proteins in CA1 areas were dissolved with lysis buffer (with PMSF) for 1 hour on ice. The protein concentration was determined using Bicinchoninic Acid (BCA) method. Equal amount of protein was loaded onto an SDS-PAGE gel. The antibodies used were as follows: Rabbit anti-Wnt7a (Abcam), rabbit anti-β-catenin (Cell Signaling Technology), rabbit anti-phospho-β-catenin (Cell Signaling Technology) and rabbit anti-β-actin (Abcam). Sample bands developed on the films were quantified by Image J software. All results were normalized against β-actin.

2.5 Semi-quantitative RT-PCR

Total RNA was extracted using the RNA kit (Axygen,USA) from the hippocampus of Pb^{2+} exposed rats or without Pb^{2+} exposure, and 10 mg/kg, 25 mg/kg and 50 mg/kg EGCG treatment. The primer Oligo dT was used to complete the reverse transcription reaction into cDNA according to the instructions (Trans Gene, China).

The 20 μL reaction pool of RT-PCR was composed of : 2 μL of 10X buffer; 0.4 μL of Taq DNA Polymerase; 1.6 μL of dNTP; 0.8 μL of forward and reverse primer; 1 μL of cDNA template and 13.4 μL of deionized water. The primers used in this protocol were listed as follows: CCAGTTCAAACCTCGCCATTAG-AAGGAATCAGCCATACATCGTG for Wnt7a and CCTGAAGTACCCCATTGA AC -GAGGTCTTTACGGATGTCAAC for β-actin. The reaction procedure was set as one cycle of 94 ◦C for 5 min, 30 cycles of 94 ◦C 30 s, 57 ◦C 30 s, 72 ◦C 1 min 40 s followed by the dissociation stage of 72 ◦C 10min. The Wnt7a mRNA levels were normalized to β-actin under the same condition.

2.6 Neuronal cultures and Small Interfering Wnt7a RNA Transfection

In order to determine whether the protective effect of EGCG on spine formation is through Wnt7a, Pb^{2+} exposed cultured hippocampal neurons were transfected with Wnt7a shRNA (Biomics Biotechnologies, Guangzhou, China) using Lipofectamine 2000 at DIV 9[23]. Then neurons were treated with EGCG (50 μM) for 48 hours and fixed with 4% paraformaldehyde (PFA) at DIV 12[24]. Neuronal images were acquired

by Olympus confocal microscope (FV1000, BX61WI). For analysis, about 3-5 dendritic segments were examined in each neuron as the Golgi-cox staining.

2.7 Data analysis

All values were given as mean ± SEM. Statistical significance of differences among groups was examined using one-way ANOVA analysis followed by Bonferroni test as post hoc analysis. $P < 0.05$ indicated a significant difference.

3. RESULTS

3.1 The concentration of Pb^{2+} in rat hippocampus

The Pb^{2+} concentration in hippocampus was 0.025 ± 0.002 μg/g in control group and 0.407 ± 0.10 μg/g in Pb^{2+}-exposed group. The Pb^{2+} concentrations in Pb^{2+}-exposed group was significantly higher than that in control ($p<0.001$) (Figure 1). The Pb^{2+} concentration in all EGCG groups did not show significant difference with Pb-exposed group ($p>0.05$).

3.2 Effect of EGCG on the spine density in hippocampal CA1 areas of Pb^{2+}-exposed rats

To explore the protective effect of EGCG on spine formation, the spine density in hippocampal CA1 areas was measured. Compared to control group, Pb^{2+}-exposed group exhibited a decrease in dendritic spine density in hippocampal CA1 pyramidal neurons (27%, $p<0.001$, Figure 2). After 10 mg/kg and 25 mg/kg EGCG

administrations, the dendritic spine density obviously increased by about 10.68% and 21.98% compared to Pb^{2+}-exposed group ($p<0.01$, $p<0.001$). However, 50 mg/kg EGCG failed to affect spine density.

3.3 Effect of EGCG on the spine width in hippocampal CA1 areas in Pb^{2+}-exposed rats

Furthermore, the head width of mushroom-like spine was measured in order to explore the effect of EGCG on spine maturation. As shown in Figure 3, Pb^{2+}-exposed group exhibited a decrease in head width of spine in hippocampal CA1 areas (control, 0.59 ± 0.018 μm; Pb^{2+}, 0.41 ± 0.013 μm; $p<0.001$) and EGCG (10 and 25 mg/kg) significantly reversed the alteration of spine head width (10 mg/kg, 0.52 ± 0.019 μm; Pb^{2+}+EGCG 25 mg/kg, 0.53 ± 0.027 μm; $p<0.01$, Figure 3). However, 50 mg/kg EGCG failed to interfere with the spine maturity after Pb^{2+} exposure.

3.4 Effect of EGCG on the Wnt7a expression in hippocampal CA1 areas in Pb^{2+}-exposed rats

Given that Wnt proteins involved in synapse formation and synaptic strength, Wnt7a expressions were analyzed by Western blotting on hippocampal CA1 areas. As shown in Figure 4, after Pb^{2+} exposure, the relative Wnt7a expression was decreased by about 10.89% in CA1 areas ($p<0.001$). After 10 mg/kg and 25 mg/kg EGCG treatment, it was significantly increased by about 6.36% and 7.15% ($p<0.01$), while 50 mg/kg EGCG failed to cause significant change.

To investigate effect of EGCG on Wnt7a gene expression, the total RNA was extracted from the rat hippocampus for Semi-quantitative RT-PCR analysis. As shown in Figure 5, the expression of Wnt7a mRNA significantly decreased 54.98% with chronic Pb^{2+} exposure ($p<0.05$). After 10 mg/kg and 25 mg/kg EGCG treatment, the level of Wnt7a mRNA were increased 56.23% and 55.42% compared to Pb^{2+} exposed group ($p<0.05$). The results provided transcription level change of Wnt7a agents after EGCG treatment, which is consistent with the translation effect.

3.5 Effect of EGCG on the expression of Wnt pathway components in hippocampal CA1 areas in Pb^{2+}-exposed rats

β-catenin plays a key role in the canonical Wnt pathway [34]. In absence of Wnt, it is phosphorylated and then ubiquitin for protectorate degradation. To further determine the activity of Wnt pathway, the level of phosphorylation of β-catenin in hippocampus was examined. Pb^{2+} exposure significantly increased the expression of phosphorylated β-catenin by 36.50% in CA1 areas ($p<0.01$, Figure 6). After 10 mg/kg and 25 mg/kg EGCG treatment, the phosphorylation level of β-catenin was significantly decreased about 22.71% and 33.32% ($p<0.05$, $p<0.01$, Figure 6), but 50 mg/kg EGCG did not have this effect.

3.6 Loss of Wnt7a expression, EGCG could not invert the impairment of spine formation after Pb^{2+} exposure

To examine Wnt7a involvement in the protection of EGCG, We then applied Wnt7a

shRNA to the cultured hippocampal neurons. Compared to control group, 1 μM Pb^{2+} can be generated to reduce the density of the spine (23.6%, $p<0.05$, Figure 7). After EGCG (50 μM) treatment, the dendritic spine density obviously increased by about 27% compared to Pb^{2+}-exposed group ($p<0.05$). However, Compared with EGCG treated group, spine was decreased 29.6% when Wnt7a shRNA was added ($P<0.01$, Figure.7). Our results showed that Wnt7a shRNA prohibited the EGCG-induced spine density increasing. These data demonstrated that Wnt7a played an essential role in spine formation.

4. DISCUSSION

Environmental lead exposure has been implicated in the impairment of synaptic plasticity and spine formation in hippocampus [23]. Our present study firstly showed that EGCG treatment could obviously inhibited decrease of spine density and maturity in hippocampal CA1 areas after developmental Pb^{2+} exposure. Meanwhile, effect of EGCG was within a certain doses and 25 mg/kg dosage presented a higher potential to recover Pb^{2+}-induced morphological damage in spine. We also found that EGCG up-regulates the activity of Wnt/β-catenin signaling pathway to reverse the impairment of spine morphology. Once the expression of Wnt7a was blocked by Wnt7a shRNA, EGCG did not exert any protective effect on Pb^{2+}-induced damages on hippocampal neurons.

The developing nervous system is sensitive to environmental insults[35]. Pb^{2+} exposure could reduce overall cognitive function in children. It is well known that

Pb^{2+} exposure during development significantly reduces hippocampal-dependent LTP [36,16], which is viewed as a key model for neuronal plasticity and memory. LTP induction elicits additional synapses formation between activated axon terminals and dendritic spines, and LTP is associated with the growth and stabilization of newly emerging dendritic spines[37]. Our previous study has shown that developmental Pb^{2+} exposure significantly decreases the spine formation in hippocampal CA1 areas, which explain the Pb^{2+}-related impairment of LTP induction. Moreover, the mature spine is characterized as a "mushroom-like" shape with thin necks and bulbous heads, which makes contact with the presynaptic terminal for signaling transmission[38, 39]. And the changes in spine shape such as shortening and/or widening of the neck would reduce its electrical resistance and thus increase strength of synapses. Our present study firstly indicates that EGCG obviously reverse the drop in spine density and mushroom head width of dendritic spines after Pb^{2+} exposure. It implies that EGCG could improve spine formation and maturity to refine synaptic efficacy in Pb^{2+}-exposed rats. To some extent, this change trend is consistent with protective effect of EGCG on LTP induction in Pb^{2+}-treated hippocampus.

The possible mechanisms of lead-related CNS impairment have been recognized as the imbalance of pro-oxidant/antioxidant and the disturbance of calcium ion[17, 18]. It has been reported that EGCG is in favor of the treatment of the neurodegenerative diseases such as PD, AD, and reduces focal ischemia/reperfusion-induced injury in CNS by its antioxidative and ion chelating properties[8, 9, 40]. In the present study, we found that EGCG could slightly decrease the lead accumulation in the developing

brain in rats. It implied that EGCG may reverse the Pb^{2+}-related impairment in the hippocampus through its antioxidative activity not its chelating property[24]. Compelling evidence has demonstrated that natural compounds with antioxidant properties have anti-inflammatory and neuroprotective effects for preventing seizure-induced pathology[41, 42]. Among these compounds, vitamin E (as α-tocopherol) also has been confirmed to reduce the spine loss in seizure [43]. Our present study firstly showed that EGCG, a kind of antioxidants, also had a high potential for improving the dendritic spines morphological stability in hippocampal CA1 areas after Pb^{2+} exposure. It provides structural evidence for our previous physiological study that EGCG significantly rescued the LTP impairment in hippocampal CA1 area in the developing Pb^{2+}-exposed rats[24]. Interestingly, our results showed that 25mg/kg seems to have better protective effect than 10 and 50mg/kg on improving the spine density and mature spine size stability in the damaged hippocampal CA1 areas. These results showed that EGCG exerts the protective function within a certain dose in chronic Pb^{2+} -exposed rats, which is consistent with our previous study[24]. The underlying mechanism of the dose-dependent manner in our present study also should be explored in our future work.

It has been well established that Wnt signaling plays an important role in the synaptic formation, growth and function[44] and Wnt/β-catenin pathway disorder has been implicated in neurodegenerative and psychiatric diseases[45]. We found that developmental Pb^{2+} exposure significantly decreased the expression of Wnt7a and increased the phosphorylation levels of β-catenin, which suggesting a decreasing

activity of Wnt/β-catenin pathway. Furthermore, one week administration of EGCG (10 and 25 mg/kg) recovered the alteration of Wnt/β-catenin pathway activity. After blocking the expression of Wnt7a to decrease the pathway activity, EGCG could not reverse the impairment of spine formation for Pb^{2+} exposure. These results suggest that EGCG could protect the synaptic plasticity of spiny neurons in hippocampus, which involving the activation of Wnt/β-catenin pathway. However, the exact mechanism underlying this effect remains to be further investigated in the future studies.

In conclusion, our present study demonstrated that developmental Pb^{2+} exposure resulted in a significant decrease of spine density and spine maturation in hippocampal CA1 areas. Importantly, we also provided evidence of neuroprotective action of EGCG against Pb^{2+}-induced spine morphological alteration. Furthermore, the present study also suggests that Wnt7a is an important target for developing spine formation, through modulating expression of key molecular in Wnt/β-catenin pathway.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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FTGURE LEGENDS

Figure 1 Lead concentration in hippocampus in the control, Pb^{2+}-exposed and Pb^{2+}+ EGCG rats. Data are represented as mean ± SEM. *** $p<0.001$.

Figure 2 Effect of EGCG on the dendritic spine density of pyramidal neurons (CA1

areas) in Pb^{2+}-exposed rat hippocampus.

Left: Dendritic branch and spines stained with the Golgi-Cox (Scale bar = 10 μm); Right: Histogram plot shows changes of dendritic spine density (spines/10 μm). Data are expressed as mean ± SEM. **p<0.01, ***p<0.001.

Figure 3 Effect of EGCG on the width of mushroom type spines of pyramidal neurons (CA1 areas) in Pb^{2+}-exposed rat hippocampus.

Histograms plot shows alteration of width of dendritic spines following chronic Pb^{2+} exposure with or without EGCG. Data are expressed as mean ± SEM. **p<0.01 and ***p<0.001.

Figure 4 Effect of EGCG on the Wnt7a expression in Pb^{2+}-exposed hippocampal CA1 areas.

Representative Immunoblot and histograms plot shows the expression of Wnt7a in CA1 areas which was normalized to β-actin. Data were expressed as mean ± SEM. ** p<0.01, *** p<0.001.

Figure 5 Effect of EGCG on the Wnt7a mRNA relative levels in hippocampus following chronic Pb^{2+} exposure.

Wnt7a mRNA expressions were evaluated by RT-PCR, quantification shows the expression of Wnt7a mRNA in hippocampus which was normalized to β-actin. Data were expressed as mean ± SEM. * p<0.05.

Figure 6 Effect of EGCG on the expression of phosphorylated β-catenin in Pb^{2+}-exposed hippocampal CA1 areas.

Representative Immunoblot and Quantification shows the ratio of expression of phosphorylated β-catenin to total β-catenin in CA1 areas. Data are expressed as mean ± SEM. *P<0.05, ** p<0.01, *** p<0.001.

Figure 7 Effect of EGCG on the spine formation after Pb^{2+} exposure in cultured hippocampal neurons with Wnt7a shRNA.

Histograms plot shows the changes of dendritic spine density in hippocampus cells after stable transfection with Wnt7a shRNA and EGFP (spines/10 μm). Data are expressed as mean ± SEM. *p<0.05, **p<0.01.

Figure 1

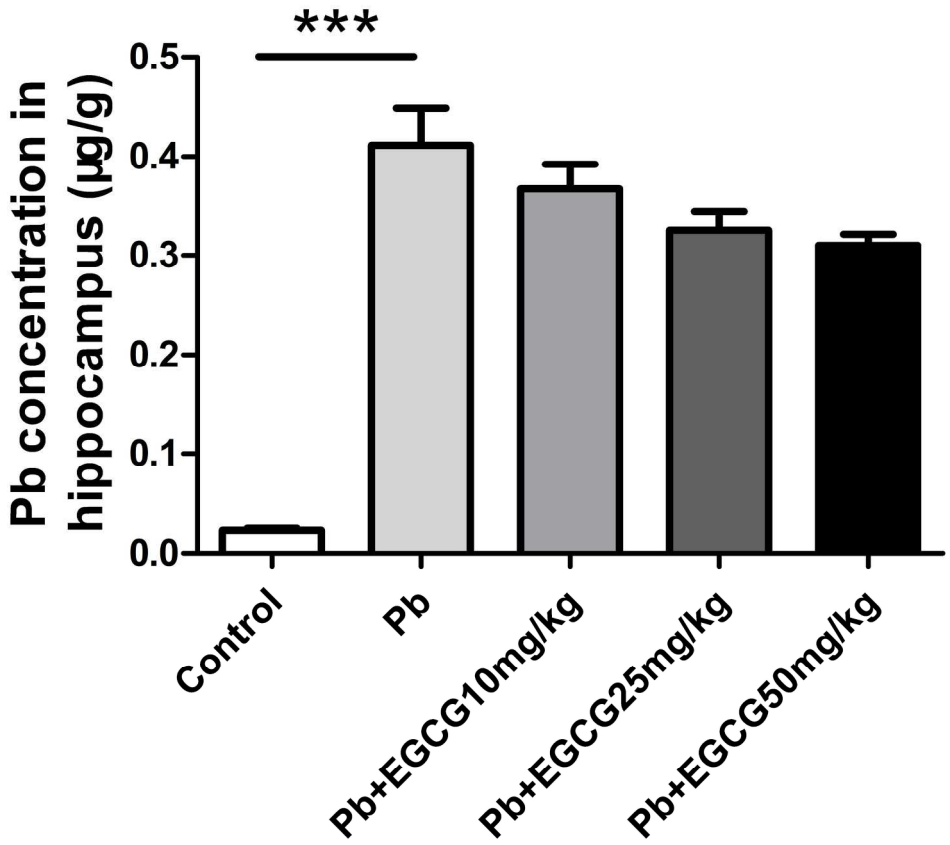

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Figure 2

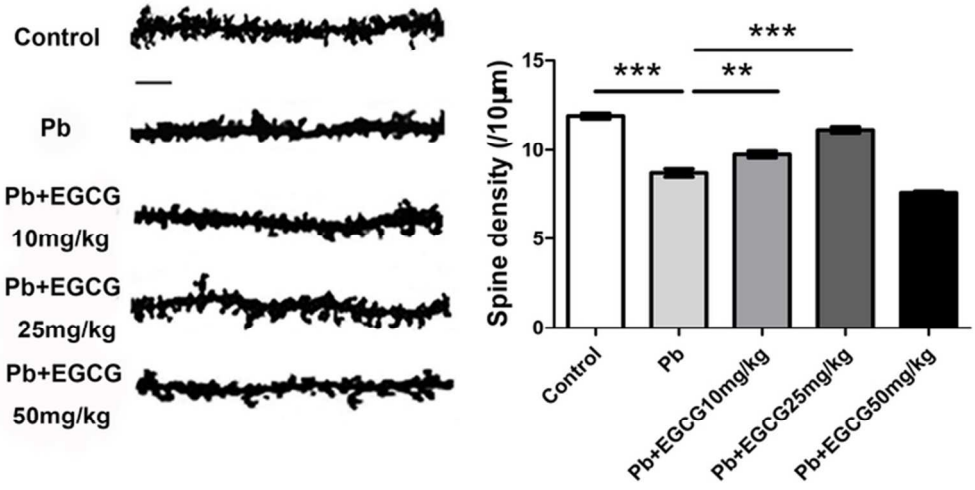

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Figure 3

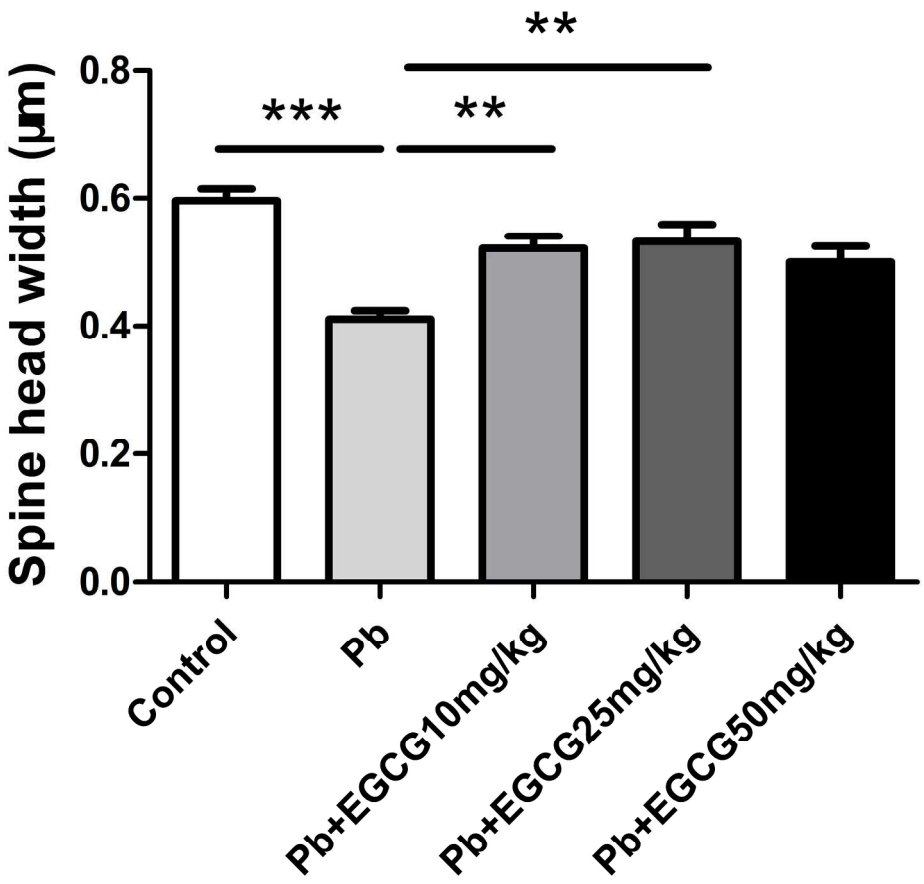

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Figure 4

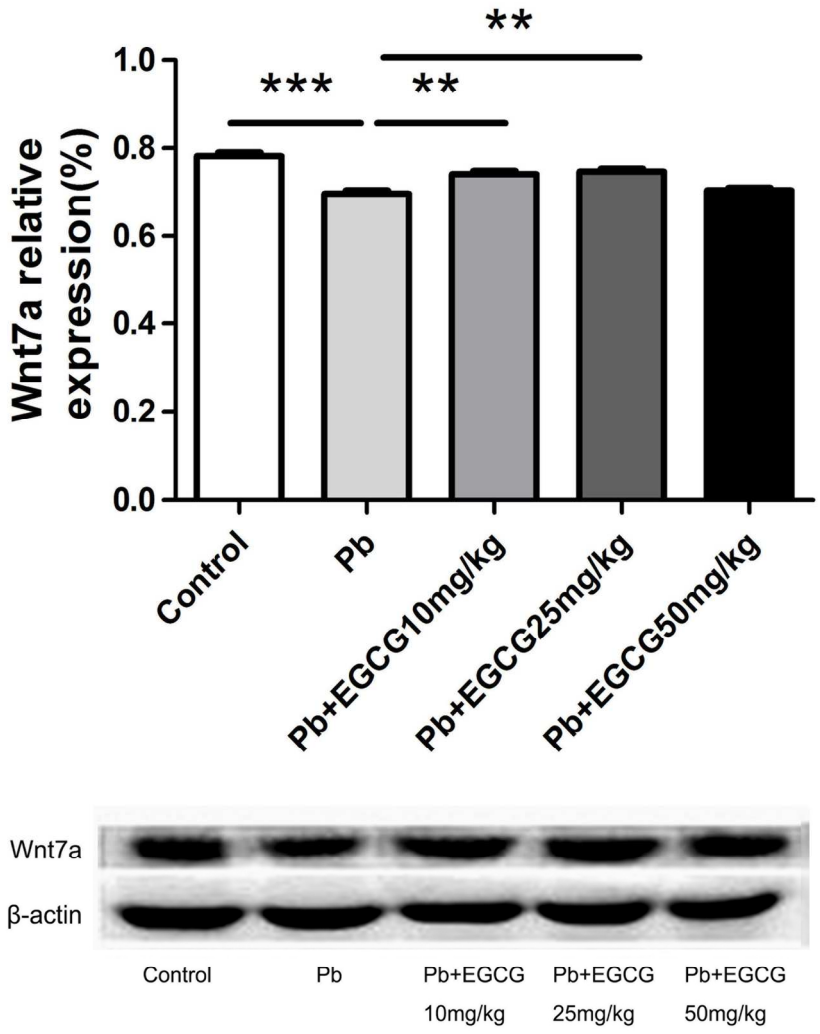

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Figure 5

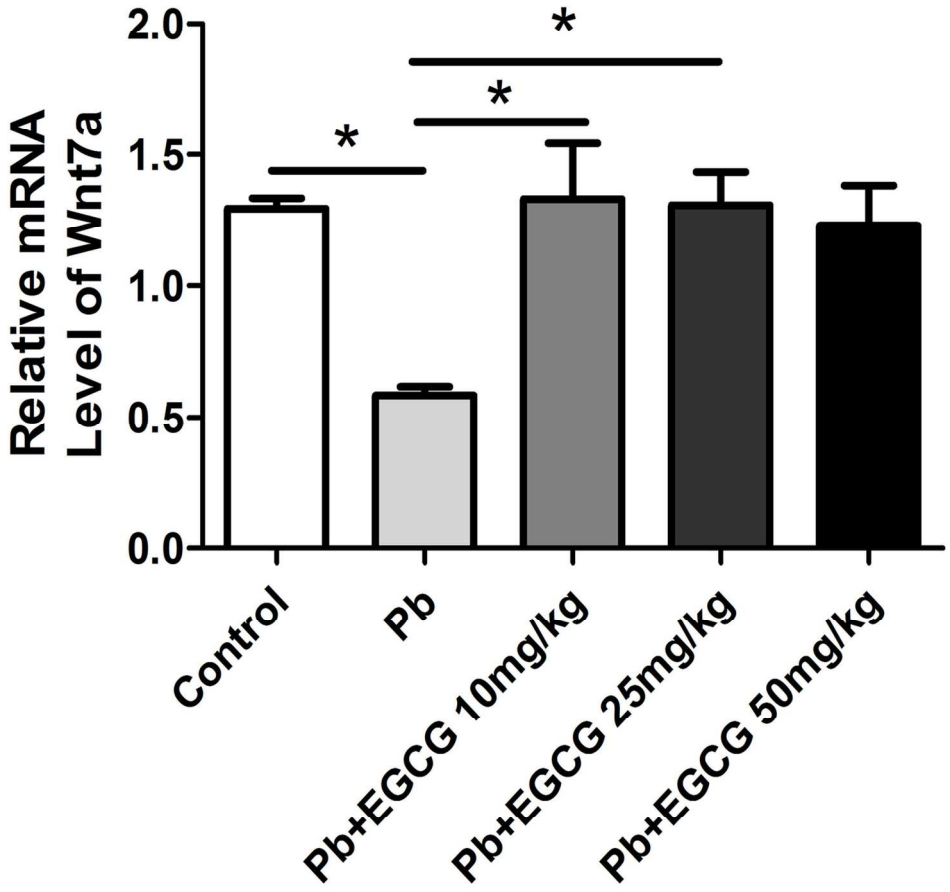

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Figure 6

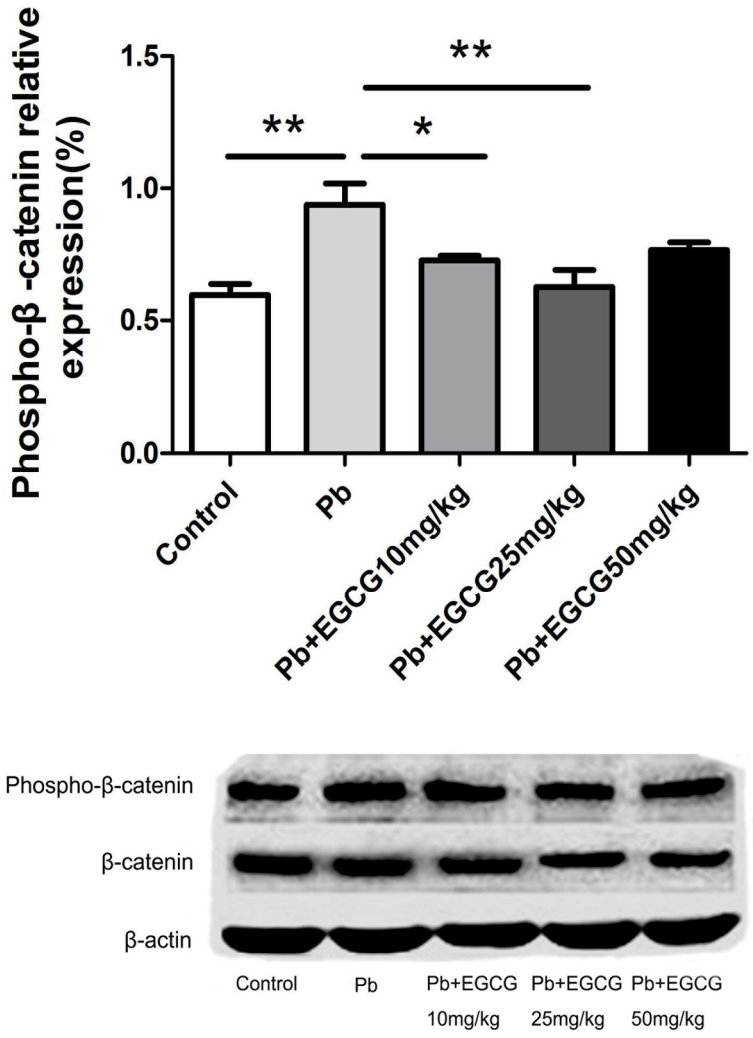

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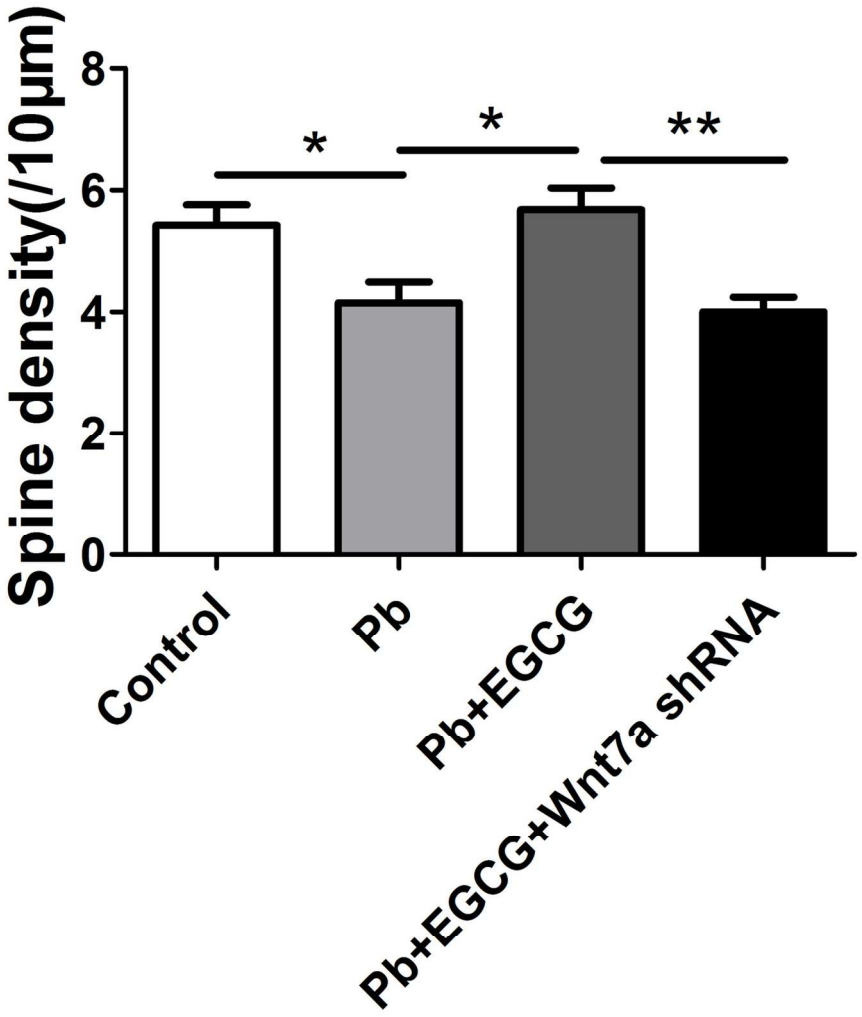
Figure 7



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