Ginsenoside Rb1 alleviates aluminum chloride-induced rat osteoblasts dysfunction¹

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Abstract: Osteoblasts dysfunction, induced by aluminum (Al), plays a critical role in the osteoporosis etiology. Ginsenoside Rb1 (Rb1) has the therapeutic properties for osteoporosis. This study aimed to assess the efficiency of Rb1 in ameliorating Al-induced osteoblasts dysfunction. The osteoblasts were divided into four groups: Rb1-treated group (RG, 0.0145 mg/mL Rb1), control group (CG, 0), AlCl₃-treated group (AG, 0.126 mg/mL AlCl₃ • 6H₂O), AlCl₃+Rb1-treated group (ARG, 0.0145 mg/mL Rb1 and 0.126 mg/mL AlCl₃•6H₂O). After 24 h of culture, the osteoblasts viability, the transforming growth factor-β1 (TGF-β₁), bone morphogenetic protein-2

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(BMP-2), the insulin-like growth factor I (IGF-I), core-binding factor $\alpha 1$ (Cbfa1) mRNA expressions, glutathione peroxidase (GSH-P_x) and superoxide dismutase (SOD) activities, and reactive oxygen species (ROS) concentration were determined. The osteoblasts ultrastructural features were also observed. In the ARG, the osteoblasts viability, TGF- β_1 , BMP-2, IGF-I and Cbfa1 mRNA expressions and the GSH-P_x and SOD activities were significantly increased, the ROS concentration was significantly decreased, and osteoblasts histology lesion was attenuated compared with the AG. These results demonstrated that Rb1 could significantly reverse osteoblasts viability and osteoblasts growth regulation factor, inhibit oxidative stress, and attenuate histology lesion in the osteoblasts with AlCl₃. These results indicate that Rb1 can effectively alleviate the AlCl₃-induced osteoblasts dysfunction.

Key words: Aluminum, Osteoblasts viability, Oxidative stress, Growth regulation factors, Ultrastructural features

1. Introduction

Aluminum (Al) is a toxic metal and Al exposure is increasing in recent days. Al is actually suspected as a factor in some cases of osteoporosis (wood et al., 2013). The mechanism of Al-induced lesion in bone is gradually clarified. Firstly, Al accumulation is the premise. It is reported that Al accumulates in the femoral head samples from patients under and over 60 years of age (Ziola-Frankowska et al., 2015), and the 21/30 patients with exostosis (Chappard et al., 2015). Secondly, the inhibition of Al on the bone formation is the main reason. Al acetate has mitodepressive effect on the bone-marrow cells (D'Souza et al., 2014). Evidence confirm that bone

formation is inhibited by long-term aluminum trichloride (AlCl_3) exposure in young growing rats (Li et al., 2011). Thirdly, The negative regulation of Al on the bone stimulatory growth factors is also important reason. The bone stimulatory growth factors has been inhibited by Al exposure in rats (Li et al., 2015). The bone has been impaired by AlCl_3 through downregulation of the Wnt/ β -catenin signaling pathway *in vivo* in young growing rats (Sun et al., 2015). The rat osteoblastic differentiation and mineralization *in vitro* has been inhibited through Wnt/ β -catenin signaling pathway (Cao et al., 2016), and TGF- β 1/Smad signaling pathway (Sun et al., 2016). It indicates that osteoblasts play a key role, and will be the target in the therapeutic of Al-induced bone lesion.

The therapeutic treatment for Al-induced toxicity has been explored. AlCl_3 -induced neurotoxicity has been ameliorated by hesperidin and silibinin through modulating of antioxidants and inflammatory cytokines contents in mice hippocampus (Jangra et al., 2015). Al sulfate-induced rat renal toxicity has been restored by melatonin (Karabulut-Bulan et al., 2015). AlCl_3 -induced nephrotoxicity has been alleviated by spirulina platensis in rats, when the animals are pre-treated (Saber et al., 2015). The Al-induced hepatic toxicity, neurotoxicity, and nephrotoxicity have been alleviated, but the Al-induced bone toxicity is not focused on.

As one of the herbal medicine, ginseng has been demonstrated to reverse bone loss and promoted bone formation. The radiation-induced bone loss and dexamethasone-induced trabecular bone loss has been prevented by the Korean red ginseng in the mice (Lee et al., 2013; Kim et al., 2015). Ginseng included the Korean red ginseng has a large number of active ingredients, commonly known as ginsenoside. Ginsenosides have the therapeutic properties for osteoporosis (Siddiqi et al. 2013). The proliferation of osteoblast has been promoted by ginsenoside Rb1 (Rb1)

(1.12×10^{-9} mol/L to 1.12×10^{-5} mol/L), and the osteoblast differentiation has been enhanced by Rb1 (1.12×10^{-7} mol/L and 1.12×10^{-6} mol/L) after 72 h of culture (Wang et al., 2010). The osteoblasts proliferation and differentiation have been promoted by different concentration of Rb1 (10^{-7} , 10^{-6} , and 10^{-5} mol/L) (Wang et al., 2014). It indicates that Rb1 has the acceleration effect on the osteoblasts proliferation and differentiation. The proliferation and apoptosis in vincristine-induced mice bone marrow stromal cells has been reversed by Rb1 (Wen et al., 2011; Hu et al., 2014). It indicates that Rb1 has the potential value in the therapeutic treatment of Al-induced bone toxicity through promoting the bone formation by osteoblasts.

Based on these findings, we hypothesized that Rb1 would be a novel strategy to protect osteoblasts suffering from Al exposure. This study was aimed to test this hypothesis; the effects of Rb1 on the osteoblasts viability, growth regulation factors (the transforming growth factor- β 1(TGF- β ₁), bone morphogenetic protein-2(BMP-2), the insulin-like growth factor I (IGF-I), core-binding factor α 1 (Cbf α 1) mRNA expressions), oxidative stress (glutathione peroxidase (GSH-P_x), superoxide dismutase(SOD) activities and reactive oxygen species (ROS) concentration) and ultrastructural features were determined in the rat osteoblasts with AlCl₃.

2. Materials and methods

2.1 Experimental protocol

The ethical protocol was approved by the Ethics Committee on the Use and Care of Animals, Institute of Special Animal and Plant Sciences of Chinese Academy of Agricultural Sciences, China. The healthy 3-day-old newborn Wistar rats were anesthetized by ether, disinfected in 75%

ethanol for 10 min. Osteoblasts were aseptically isolated from the skull of the rats according to Li et al. (2012a). As the Al doses used in different groups were different, $1/10IC_{50}$ $AlCl_3 \cdot 6H_2O$ was chosen to design the treatment experiment with Rb1. The dose of Rb1 was chosen according to Wang et al. (2010) and Wang et al. (2014). Though the chosen Al dose was higher than human exposure dose. If the toxic effects of $1/10IC_{50}$ $AlCl_3 \cdot 6H_2O$ were restored by the Rb1, the lower dose of $AlCl_3 \cdot 6H_2O$ than this will also be treated.

The osteoblasts were plated at a concentration of 5×10^5 cells/mL in cell culture flask (25 cm²) with DMEM/10% FBS. The osteoblasts were divided into four groups (n=10): Rb1-treated group (RG, 0.0145 mg/mL Rb1, pH=6.5), control group (CG, 0), $AlCl_3$ -treated group (AG, 0.126 mg/mL $AlCl_3 \cdot 6H_2O$, pH=5.5), $AlCl_3$ +Rb1-treated group (ARG, 0.0145 mg/mL Rb1 and 0.126 mg/mL $AlCl_3 \cdot 6H_2O$, pH=6.0). After 24 h, the osteoblasts were used to determine osteoblasts viability, TGF- β_1 , BMP-2, IGF-I, *Cbfa1* mRNA expressions, GSH-P_x, SOD activities and ROS concentration in the each group. The osteoblasts ultrastructural features were also observed.

2.2 The osteoblasts viability determination

The osteoblasts viability in the RG, CG, AG and ARG were determined using cell counting Kit 8 (CCK8 Kit, Dojindo Molecular Technologies Inc., Gaithersburg, Md) by the protocol.

2.3 The osteoblasts TGF- β_1 , BMP-2, IGF-I and Cbfa1 mRNA expressions measurement

The osteoblasts mRNA in the each group was extracted using Easy Pure RNA Kit (Beijing TransGen Biotech Co., Ltd) by the protocol. The mRNA was reverse transcribed into cDNA using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (Beijing TransGen Biotech

Co., Ltd) by the protocol. The cDNA was stored at -80 °C. Specific primers for TGF- β_1 , BMP-2, IGF-I, Cbfa1 and β -actin gene were designed using Primer Premier 5.0 software and were synthesized by Sangon Biotech (Shanghai, China) (Tab. 1). Then the cDNA was amplified using LightCycler® 96 real time fluorescence ration PCR instrument (Roche, Switzerland) with TransStart Top Green qPCR SuperMix (Beijing TransGen Biotech Co., Ltd) by the protocol.

2.4The osteoblasts GSH-P_x, SOD activities and ROS concentration measurement

The GSH-P_x, SOD activities and ROS concentration in the osteoblasts were determined using Glutathione Peroxidase (GSH-P_x) assay kit (Colorimetric method), Total Superoxide Dismutase (T-SOD) assay kit (Hydroxylamine method) and Reactive oxygen species Assay Kit (chemistry fluorescence method) from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the protocol, respectively.

2.5Osteoblasts histopathological analysis

The osteoblasts in the RG, CG, AG and ARG were fixed for three days in a glutaraldehyde/formaldehyde solution (Sigma) and washed for 24 h in cacodylic acid buffer solution (pH 7.0-7.2). Osteoblasts were treated with osmium 1% (Merck, Darmstadt), washed several times with cacodylic acid buffer solution and dehydrated with alcohol. Then, the osteoblasts were transferred into pure propylenoxide, which were replaced with a ratio of 3:1 mixture of propylenoxide and Polybed® 812 (Embedding Media/DMP-30, Polysciences Inc., Warrington, USA) followed by a ratio of 1:1 and 1:3 mixture, respectively. The blocks were incubated in a pure Polybed® 812 solutions for 12 or 24 h. Polymerisation was completed after

two days at 40°C and four days at 70°C. To observe the osteoblast ultrastructural features, 1 µm thin-sections were sliced from the hardened blocks with an ultra-mikrotom (Ultra-Cut, S Reichert-Jung), dyed with uranyl acetate and lead citrate, put on copper nets and analyzed with a transmission electron microscope T-400 (Philips, Eindhoven, NL).

2.6 Statistical analysis

Statistical analysis were done using SPSS 22.0 package programmer (SPSS Inc., Chicago, IL, USA). Shapiro-Wilk test was used to check the normal distribution of data. Levene's test was used to assess the variance homogeneity. One-way ANOVA with LSD and Bonferroni's method was used to conduct multiple comparisons. Data were shown as least square means and standard deviation. $P < 0.05$ was considered significant difference, and $P < 0.01$ was considered markedly significant difference.

3. Results

3.1 The Rb1 alleviated AlCl₃-induced inhibition of the osteoblasts viability

The osteoblasts viability decreased in the AG and increased in the RG and ARG. The osteoblasts viability was lower in the AG and higher in the RG compared with the CG ($P < 0.05$), and that in the ARG ($P < 0.01$) was higher compared with the AG (Fig.1).

3.2 The Rb1 alleviated AlCl₃-induced inhibition on the osteoblasts growth regulation factors mRNA expressions

As shown in Fig.2, the TGF- β_1 , BMP-2, IGF-I and Cbfa1 mRNA expressions decreased in the AG and increased in the RG and ARG. The TGF- β_1 , BMP-2, IGF-I and Cbfa1 mRNA expressions were lower in the AG ($P < 0.01$) and higher in the RG ($P < 0.05$; $P < 0.01$) compared with the CG, and those in the ARG ($P < 0.01$) were higher compared with the AG.

3.3 The Rb1 attenuated the AlCl₃-induced oxidative stress in the osteoblasts

The GSH-P_x and SOD activities decreased in the AG and increased in the RG and ARG. The GSH-P_x and SOD activities were lower in the AG ($P < 0.01$) and higher in the RG ($P > 0.05$) compared with the CG, and those in the ARG ($P < 0.01$) were higher compared with the AG. The ROS concentration increased in the AG and decreased in the RG and ARG. The ROS concentration was higher in the AG ($P < 0.01$) and lower in the RG ($P < 0.05$) compared with the CG, and that was lower in the ARG ($P < 0.01$) compared with the AG (Fig.3).

3.4 The osteoblasts ultrastructural features

Observations of the osteoblasts ultrastructural features in the CG and RG revealed intact cell membrane, dispersed chromatin, normal morphology of mitochondria and endoplasmic reticulum (Fig.4.A, D). Observations of the osteoblasts ultrastructural features in the AG revealed that chromatolysis (Fig.4.B.a), incomplete nuclear membrane (Fig.4.B.b), lots of foam-like structure (Fig.4.B.c), mitochondrial swelling, spinal fracture (Fig.4.B.d), incomplete cell membrane and cytoplasm spillover (Fig.4.B.e). The osteoblasts ultrastructural features in the ARG were restored by Rb1 (Fig.4. C). The corresponding quantification of histological data were showed in Tab 2.

4. Discussion

The finding of the present study suggests that Rb1 attenuates the AlCl_3 -induced lesion in the osteoblasts viability and the ultrastructural features through suppressing the oxidative stress. Rb1 alleviates the the AlCl_3 -induced lesion in the osteoblasts growth regulation factors through repairing the osteoblasts viability and ultrastructural features.

The extracellular signal-regulated kinase (ERK), the JNK and p38 activation and the phosphatidylinositol 3-kinase (PI3K) /AKT pathway plays an important role in the cell death. ERK mRNA expression has been reduced by Al (Ge et al., 2007). The phosphorylation state of JNK has been increased by Al (Li et al., 2012a). And autophagy-related protein (Beclin 1 and LC3II) expressions has been induced by Al (Zeng et al., 2012). The inhibition of the ERK-MAPK, PI3K/AKT and JNK signaling pathway may be the key for AlCl_3 -induced lower osteoblasts viability. The hypoxia-reoxygenation-induced apoptosis in H9c2 cardiomyocytes has been prevented by Rb1 among the AKT, JNK, and ERK 1/2 pathways (Ai et al., 2015). The treatment of Rb1 on the AlCl_3 -induced osteoblasts viability may depend on its effect on the ERK-MAPK, PI3K/AKT and JNK signaling pathway.

TGF- β_1 , BMP-2, IGF-I and Cbfa1 are osteoblasts growth regulation factors and cooperate to regulate the osteoblasts function. TGF- β_1 enhances bone regeneration (Farea et al, 2014) and promotes the synthesis of type I collagen from the osteoblasts. Type I collagen is responsible for bone stability and cell biological functions (Reis et al., 2015). It indicates that Rb1 promotes bone stability and cell biological functions. BMP-2 regulates *in vitro* osteogenic differentiation of mouse adipose derived stem cells (Chen et al., 2015). Loss of BMP-2 and -4 has been induced

severe osteogenesis impairment (Pizette and Niswander, 2001). IGF-I regulates the location, stability, and transcriptional activity of β -catenin (Playford et al., 2000). The activation of Wnt/ β -catenin signaling pathway plays vital role in the osteoblasts proliferation and differentiation (Issack et al., 2008). Cbfa1 is one of the osteogenic master transcription factors (Phimphilai et al., 2006). Transgenic research shows that Cbfa1 can directly combine with promoter region in the type I collagen protein, osteocalcin, osteopontin gene (Cui et al., 2012). Type I collagen, osteocalcin and osteopontin play important role in the osteoblasts differentiation and maturation (Ziros et al., 2002). In this study, Rb1 promote the TGF- β_1 , BMP-2, IGF-I and Cbfa1 mRNA expression in the rat osteoblasts with AlCl₃. It indicates that Rb1 promote the osteogenesis through increasing the TGF- β_1 , BMP-2, IGF-I and Cbfa1 mRNA expression.

Here we report that the ROS contents has been suppressed, and the GSH and SOD activities have been increased by Rb1 in osteoblasts with AlCl₃. The similar anti-oxidative effect of Rb1 has been found in UV-B-irradiated HaCaT cells (Oh et al., 2015) and in the H₂O₂-treated embryonic chick cardiomyocytes (Li et al. 2012b). These evidences support our results. Mitochondria are the main source of ROS and also the principal target of ROS attacks. The damaged mitochondria accumulate under oxidative stress, suggesting that maintaining healthy mitochondria is crucial for protecting against pathological change induced by AlCl₃. In the current experiment, Rb1 attenuates AlCl₃-induced osteoblasts histology lesion through suppressing oxidative stress.

In conclusions, Rb1 significantly attenuates AlCl₃-induced lesion in the osteoblasts viability, osteoblasts growth regulation factor mRNA expressions and the osteoblasts ultrastructural features, and inhibits AlCl₃-induced oxidative stress. It indicates that Rb1 can effectively alleviate the AlCl₃-induced osteoblasts dysfunction. Although further studies are required, we can still propose

Rb1 for the rescue from AlCl_3 -induced osteoblasts dysfunction.

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References

- Ai QD, Sun G.B, Luo Y, Dong, X., Hu, R.F., Meng, X.B., Sun, X.b. 2015. Ginsenoside Rb1 prevents hypoxia-reoxygenation-induced apoptosis in H9c2 cardiomyocytes via an estrogen receptor-dependent crosstalk among the Akt, JNK, and ERK 1/2 pathways using a label-free quantitative proteomics analysis. *RSC Adv.* 5, 26346-26363.
- Chappard, D., Mabilieu, G., Moukoko, D., Henric, N., Steiger, V., Le Nay, P., Frin, J.M., De Bodman, C. 2015. Aluminum and iron can be deposited in the calcified matrix of bone exostoses. *J Inorg Biochem* 152, 174-179.
- Cao, Z., Fu, Y., Sun, X., Zhang, Q., Xu, F., Li, Y. 2016. Aluminum trichloride inhibits osteoblastic differentiation through inactivation of Wnt/ β -catenin signaling pathway in rat osteoblasts. *Environ. Toxicol. Pharmacol.* 42, 198-204.
- Chen, X.D., Deng, M., Zhou, J.S., Xiao, Y.Z., Zhou, X.S., Zhang, C.C., Wu, M., Wang, Z.D., Chen, X.T. 2015. Bone morphogenetic protein-2 regulates in vitro osteogenic differentiation of mouse adipose derived stem cells. *Eur. Rev. Med. Pharmacol. Sci.* 19, 2048-2053.
- Cui, X., Wang, B., Zong, Z., Cui, X., Wang, B., Zong, Z., Liu, S., Xing, W. 2012. The effects of chronic aluminum exposure on learning and memory of rats by observing the changes of Ras/Raf/ERK signal transduction pathway. *Food Chem. Toxicol.* 50, 315-319.
- D'Souza, S.P., Vijayalaxmi, K.K., Naik, P. 2014. Assessment of genotoxicity of aluminium acetate in bone marrow, male germ cells and fetal liver cells of Swiss albino mice. *Mutat Res Genet Toxicol. Environ. Mutagen.* 766, 16-22.
- Farea, M., Husein, A., Halim, A.S., Abdullah, N.A., Mokhtar, K.I., Lim, C.K., Berahim, Z., Mokhtar, K. 2014. Synergistic effects of chitosan scaffold and TGF β 1 on the proliferation and

osteogenic differentiation of dental pulp stem cells derived from human exfoliated deciduous teeth.

Arch. Oral Biol. 59, 1400-1411.

Hu, W.X., Wen, Z., Rong, J.P., Yu, H., Hu, G.Z., He, D. 2014. Effect of ginsenoside Rb1 on preventing from the apoptosis of bone marrow stromal cells induced by vincristine. Pharm. Clinics Chin. Materia. Medica. 30, 40-43.

Issack, P.S., Helfet, D.L., Lane, J.M. 2008. Role of wnt signaling in bone remodeling and repair. HSS. J. 4, 66-70.

Jangra, A., Kasbe, P., Pandey, S.N., Dwivedi, S., Gurjar, S.S., Kwatra, M., Mishra, M., Venu, A.K., Sulakhiya, K., Gogoi, R., Sarma, N., Bezbaruah, B.K., Lahkar, M. 2015. Hesperidin and silibinin ameliorate aluminum-induced neurotoxicity: modulation of antioxidants and inflammatory cytokines level in mice hippocampus. Biol. Trace Elem. Res. 168, 462-471.

Karabulut-Bulan, O., Bayrak, B.B., Arda-Pirincci, P., Sarikaya-Unal, G., Us, H., Yanardag, R. 2015. Role of exogenous melatonin on cell Proliferation and oxidant/antioxidant system in aluminum-induced renal toxicity. Biol.Trace Elem. Res. 168, 141-149.

Kim, J., Lee, H., Kang, K.S., Chun, K.H., Hwang, G.S. 2015. Protective effect of korean red ginseng against glucocorticoid-induced osteoporosis in vitro and in vivo. J. Gens Res. 39, 46-53.

Li, X., Zhang, L., Zhu, Y., Li, Y. 2011. Dynamic analysis of exposure to aluminum and an acidic condition on bone formation in young growing rats. Environ. Toxicol. Pharmacol. 31, 295-301.

Li, P., Luo, W., Zhang, H., Zheng, X., Liu, C., Ouyang, H. 2015. Effects of Aluminum Exposure on the Bone Stimulatory Growth Factors in Rats. Biol. Trace Elem. Res. doi:10.1007/s12011-015-0569-z.

Lee, J.H., Lee, H.J., Yang, M., Moon, C., Kim, J.C., Bae, C.S., Jo, S.K., Jang, J.S., Kim, S.H.

2013. Effect of korean red ginseng on radiation-induced bone loss in C3H/HeN mice. *J. Gens. Res.* 37, 435-441.

Li, X., Han, Y., Guan, Y., Zhang, L., Bai, C., Li, Y. 2012a. Aluminum induces osteoblast apoptosis through the oxidative stress-mediated JNK signaling pathway. *Biol. Trace Elem. Res.* 150, 502-508.

Li, J., Shao, Z.H., Xie, J.T., Wang, C.Z., Ramachandran, S., Yin, J.J., Aung, H., Li, C.Q., Qin, G., Vanden Hoek, T., Yuan, C.S. 2012b. The effects of ginsenoside Rb1 on JNK in oxidative injury in cardiomyocytes. *Arch. Pharm. Res.* 35, 1259-1267.

Oh, S.J., Kim, K., Lim, C.J. 2015. Protective properties of ginsenoside Rb1 against UV-B radiation-induced oxidative stress in human dermal keratinocytes. *Pharmazie* 70, 381-387.

Playford, M.P., Bicknell, D., Bodmer, W.F., Macaulay, V.M. 2000. Insulin-like growth factor 1 regulates the location, stability, and transcriptional activity of beta-catenin. *Proc. Natl. Acad. Sci. U S A* 97, 12103-12108.

Pizette, S., Niswander, L. 2001. Early steps in limb patterning and chondrogenesis. *Novartis. Found Symp.* 232, 23-36; discussion 36-46.

Phimphilai, M., Zhao, Z., Boules, H., Roca, H., Franceschi, R.T. 2006. BMP signaling is required for RUNX2-dependent induction of the osteoblast phenotype. *J. Bone Miner. Res.* 21, 637-646.

Reis, A.M., Ribeiro, L.G., Ocarino, Nde, M., Goes, A.M., Serakides, R. 2015. Osteogenic potential of osteoblasts from neonatal rats born to mothers treated with caffeine throughout pregnancy. *BMC Musculoskel. Disord.* 16, 1-11.

Sun, X.D., Cao, Z., Zhang, Q.Y., Li, M., Han, L., Li, Y. 2016. Aluminum trichloride inhibits osteoblast mineralization via TGF- β 1/Smad signaling pathway. *Chem-Biol. Interact.* 244, 9-15.

- Siddiqi, M.H., Siddiqi, M.Z., Ahn, S., Kang, S., Kim, Y.J., Sathishkumar, N., Yang, D.U., Yang, D.C. 2013. Ginseng saponins and the treatment of osteoporosis: mini literature review. *J. Gens. Res.* 37, 261-268.
- Saber, T.M., Elgaml, S.A., Ali, H.A., Saleh, A.A. 2015. Protective effect of spirulina platensis against aluminium-induced nephrotoxicity and DNA damage in rats. *Toxicol. Environ. Chem.* 97, 1113-1123.
- Sun, X.D., Cao, Z., Zhang, Q.Y., Liu, S., Xu, F., Che, J., Zhu, Y., Li, Y., Pan, C., Liang, W. 2015. Aluminum trichloride impairs bone and downregulates Wnt/ β -catenin signaling pathway in young growing rats. *Food Chem. Toxicol.* 86, 154–162.
- Wood, C.L., Wood, A.M., Harker, C., Embleton, N.D. 2013. Bone mineral density and osteoporosis after preterm birth: the role of early life factors and nutrition. *Int J Endocrinol* 2013:902513.
- Wang, J.J., Jiang, Y.Z., Liang, J.C., Chen, Y. 2010. Effects of ginsenoside Rb1 on cell proliferation, differentiation and OPG/RANKL mRNA expression of rat osteoblast cultured in vitro. *Chin. J. Trad. Chin. Med. Pharm.* 25, 939-942.
- Wang, W., Huang, K., Zheng, L.L. 2014. Effect of different concentrations of ginseng saponin Rb1 on osteoblast proliferation and differentiation. *Chin. J. Bio. Pharm.* 34, 50-56.
- Wen, Z., Hu, W.H., Hu, G.Z., He, D., Rong, J.P.M., Yu, H. 2011. Study of ginsenoside Rb1 interfering with proliferation of bone marrow stromal cells inhibited by VCR. *Pharm. Clinics Chin. Materia. Medica.* 27, 12-14.
- Ziola-Frankowska, A., Dabrowski, M., Kubaszewski, L., Rogala, P., Frankowski, M. 2015. Factors affecting the aluminium content of human femoral head and neck. *J. Inorg. Biochem.* 152, 167-173.

Zeng, K.W., Fu, H., Liu, G.X., Wang, X.M. 2012. Aluminum maltolate induces primary rat astrocyte apoptosis via overactivation of the class III PI3K/Beclin 1-dependent autophagy signal. *Toxicol. In Vitro* 26, 215-220.

Ziros, P.G., Gil, A.P., Georgakopoulos, T., Habeos, I., Kletsas, D., Basdra, E.K., Papavassiliou, A.G. 2002. The bone-specific transcriptional regulator Cbfa1 is a target of mechanical signals in osteoblastic cells. *J. Biol. Chem.* 277, 23934-23941.

Tab.1 The primer sequence, primer length and the product length of the amplification target gene

Gene	Number	Upstream and downstream primer sequence	Primer	Product
			length (bp)	length (bp)
TGF- β_1	AY5500	F: 5' GCTAATGGTGGACCGCAACAACG 3'	23	286
	25.1	R: 5' CTTGCTGTACTGTGTGTCCAGGC 3'	23	
BMP-2	NM_085	F: 5' ACAAACGAGAAAAGCGTCAAGC 3'	22	291
	302.1	R: 5' CCAGTCATTCCACCCCACATCA 3'	22	
IGF-I	XM_006	F: 5' TGCTTGCTCACCTTTACCAG 3'	20	328
	205900	R: 5' ACATCTCCAGCCTCCTCAGA 3'	20	
Cbfa1	NM_357	F: 5' ACAAACGAGAAAAGCGTCAAGC 3'	22	323
	89.2	R: 5' CCAGTCATTCCACCCCACATCA 3'	22	
β -actin	NM_031	F: 5' TGTAACCAACTGGGACGATA 3'	18	289
	144.2	R: 5' GAGGCATACAGGGACAACA 3'	20

Fig.1 The Rb1 alleviated AlCl_3 -induced inhibition on the osteoblasts viability. Ten osteoblasts samples from the RG (Rb1 group), CG (control group), AG (AlCl_3 -treated group), and ARG (AlCl_3 +Rb1-treated group) were collected for the determination of osteoblasts viability using CCK8 method, respectively. Data were shown as least square means and standard deviation (SD, bar on the top of each column). One-way ANOVA with LSD and Bonferroni's method was used to conduct multiple comparisons. * $P < 0.05$ versus the CG, ## $P < 0.01$ versus the AG.

Fig.2 The Rb1 alleviated AlCl₃-induced inhibition on the osteoblasts growth regulation factors mRNA expressions. Three osteoblasts samples from the RG (Rb1 group), CG (control group), AG (AlCl₃-treated group), and ARG (AlCl₃+Rb1-treated group) were collected for the determination of TGF-β₁, BMP-2, IGF-I and Cbfa1 mRNA expressions, respectively. Data were shown as least square means and standard deviation (SD, bar on the top of each column). One-way ANOVA with LSD and Bonferroni's method was used to conduct multiple comparisons. ** $P < 0.01$ versus the CG, ## $P < 0.01$ versus the AG.

Fig.3 The Rb1 attenuated the AlCl₃-induced oxidative stress in the osteoblasts. Ten osteoblasts samples from the RG (Rb1 group), CG (control group), AG (AlCl₃-treated group), and ARG (AlCl₃+Rb1-treated group) were collected for the determination of the GSH-P_x and SOD activities, and ROS concentration, respectively. Data were shown as least square means and standard deviation (SD, bar on the top of each column). One-way ANOVA with LSD and Bonferroni's method was used to conduct multiple comparisons. ** $P < 0.01$ versus the CG, ## $P < 0.01$ versus the AG.

Fig.4 The histopathological change in the osteoblasts and protection offered by Rb1 (2 μ m). One μ m thin-sections from the A (CG control group), B (AG AlCl_3 -treated group), C (ARG AlCl_3 +Rb1-treated group) and D (RG Rb1-treated group) were observed by a transmission electron microscope T-400 (Philips, Eindhoven, NL). Fig.4.B.a, chromatolysm, b, incomplete nuclear membrane, c, lots of foam-like structure, d, mitochondrial swelling, spinal fracture, e, incomplete cell membrane and cytoplasm spillover.

Tab. 2 The score of the osteoblasts ultrastructural features in different groups.

Item	CG	AG	ARG	RG
The score	++++	----	++	+++

Note: “----” indicates the worse histological features, and “++++” indicates the normal histological features. “++” and “+++” indicates the histological features compared with “++++” and “----”

Figure: The Rb1 alleviated AlCl₃-induced inhibition in the osteoblasts viability (n=10). The osteoblasts samples from CG (control group), AG (AlCl₃-treated group), and RG (Rb1-treated group) were collected for the determination of osteoblasts viability using CCK8 method. * $P < 0.05$ versus the CG, ## $P < 0.01$ versus the AG.