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RESEARCH PAPER

Treatment with resveratrol attenuates sublesional bone loss in spinal cord-injured rats

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BACKGROUND AND PURPOSE

Sublesional osteoporosis predisposes individuals with spinal cord injury (SCI) to an increased risk of low-trauma fracture. The aim of the present work was to investigate the effect of treatment with resveratrol (RES) on sublesional bone loss in spinal cord-injured rats.

EXPERIMENTAL APPROACH

Complete SCI was generated by surgical transaction of the cord at the T_{10-12} level. Treatment with RES (400 mg·kg^{-1} body mass per day^{-1}, intragastrically) was initiated 12 h after the surgery for 10 days. Then, blood was collected and femurs and tibiae were removed for evaluation of the effects of RES on bone tissue after SCI.

KEY RESULTS

Treatment of SCI rats with RES prevented the reduction of bone mass including bone mineral content and bone mineral density in tibiae, preserved bone structure including trabecular bone volume fraction, trabecular number, and trabecular thickness in tibiae, and preserved mechanical strength including ultimate load, stiffness, and energy in femurs. Treatment of SCI rats with RES enhanced femoral total sulfhydryl content, reduced femoral malondialdehyde and IL-6 mRNA levels. Treatment of SCI rats with RES suppressed the up-regulation of mRNA levels of PPARγ, adipose-specific fatty-acid-binding protein and lipoprotein lipase, and restored mRNA levels of Wnt1, low-density lipoprotein-related protein 5, Axin2, ctnnb1, insulin-like growth factor 1 (IGF-1) and receptor for IGF-1 in femurs and tibiae.

CONCLUSIONS AND IMPLICATIONS

Treatment with RES attenuated sublesional bone loss in spinal-cord-injured rats, associated with abating oxidative stress, attenuating inflammation, depressing PPARγ signalling, and restoring Wnt/β-catenin and IGF-1 signalling.

Abbreviations

25(OH)D, 25-hydroxyvitamin D; aP2, adipose-specific fatty-acid-binding protein; BFR/BS, bone formation rate/bone surface; BMD, bone mineral density; BV/TV, trabecular bone volume fraction; DPD, deoxypyridinoline; ES/BS, eroded surface/bone surface; IGF-1, insulin-like growth factor 1; IGF-1R, receptor for insulin-like growth factor; IGFBP5, insulin-like growth-factor-binding-protein 5; LPL, lipoprotein lipase; Lrp5, low-density lipoprotein-related protein 5; MAR, mineral apposition rate; MDA, malondialdehyde; Oc.S/BS, osteoclast surface/bone surface; OPG, osteoprotegerin; PPARγ, peroxisome proliferator-activated receptor gamma; RANKL, receptor activator of NF-κB ligand; RES, resveratrol; SCI, spinal cord injury; TAC, total antioxidant capacity; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; TRAP, tartrate-resistant acid phosphatase; t-SH, total sulfhydryl

Introduction

One of the inevitable consequences of spinal cord injury (SCI) is the significant bone loss within a few months to a few years after trauma (Kocina, 1997). This bone loss leads to fractures in up to 50% of individuals with complete SCI; the most commonly affected sites are long bones of the lower limbs and the trabecular metaphysical-epiphyseal areas of the distal femur and proximal tibiae (Garland *et al.*, 1992; Wilmet *et al.*, 1995; Dauty *et al.*, 2000). SCI-induced bone loss carries significant morbidity and can worsen already profound disability. To prevent bone loss after SCI, many cases are treated with passive standing, low-level electrical stimulation, and body weight-supported treadmill training. However, findings with respect to the effects of these interventions on osteoporosis after SCI are less consistent. Because treatment interventions based on physical activity and electrical stimulation had no sufficient positive effects (Needham-Shropshire *et al.*, 1997; Ben *et al.*, 2005; Giangregorio *et al.*, 2005), pharmacologic therapy seemed to be necessary.

Resveratrol (RES, 3,4′,5-trihydroxystilbene) is a naturally occurring phytoalexin generated in a number of plants including grapes, mulberries, cranberries, and peanuts. Recent studies reported that RES could attenuate neural lesion in rat model of SCI (Yang and Piao, 2003; Liu *et al.*, 2011). RES has recently attracted considerable interest because of its protective effects on multiple events associated with osteoporosis. Treatment with RES delayed age-related bone loss in rats and mice (Pearson *et al.*, 2008) and protected against bone loss induced by oestrogen deficiency (Liu *et al.*, 2005). More importantly, chronic RES supplementation maintained the bone mineral density (BMD) and strength of the femur of rat hindlimb unloading (Momken *et al.*, 2011); prior treatment with RES preserved density and structure of rat long bones under tail-suspension (Habold *et al.*, 2011). It was reported that mechanical unloading might play an important role in the pathogenesis of osteoporosis after SCI (Jiang *et al.*, 2006).

In addition, it was reported that in the early stage both oversupply of osteoclasts relative to the requirement for bone resorption and undersupply of osteoblasts relative to the requirement for cavity repair were involved in the bone loss (Morse *et al.*, 2008; 2011) after SCI. RES was shown to be able to promote osteogenic differentiation of mesenchymal stem cells (MSCs; Zhou *et al.*, 2009; Shakibaei *et al.*, 2012) and inhibit receptor activator of NF-κB ligand (RANKL)-induced osteoclast differentiation of both bone-derived cells and osteoclast progenitor RAW 264.7 cells (He *et al.*, 2010; Shakibaei *et al.*, 2011).

The aim of our study was thus to test RES as a preventive countermeasure against bone loss induced by SCI in rats.

Methods

Animals

Sprague-Dawley (SD, male, 6 weeks old) rats were purchased from the Vital-Aiver Animal Ltd (Beijing, China). All the rats were fed under controlled temperature (21–23°C), 12 h light and 12 h dark cycles (light, 0800–2000 h; darkness, 2000–0800 h), and free access to food and tap water. All the animals used in this study received humane care in compliance with institutional animal care guidelines. All the surgical and experimental procedures were in accordance with institutional animal care guidelines, and were approved by the Local Institutional Committee. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Chemicals, drugs and reagents were obtained from Sigma Chemical (St. Louis, MO, USA) unless otherwise stated.

The drug/molecular target nomenclature used in this manuscript conforms to British Journal of Pharmacology's Guide to Receptors and Channels (Alexander *et al.*, 2011).

Animal model of SCI

All rats were anaesthetized by intraperitoneal injection of xylazine (10 mg·kg^{-1} body mass) and ketamine (75 mg·kg^{-1} body mass). With the back shaved and sterilized, an incision was made on the back posterior to the lower thoracic region. After the back muscles were infiltrated, the dorsal surface of the spinal cord was exposed by laminectomy at the $T_{10–12}$ levels, and the lower thoracic cord was subsequently completely transected with fine scissors. Two surgeons independently verified a complete transection by passing a glass probe through the site and lifting both cut ends of the spinal cord. A collagen matrix, Vitrogen, was injected into the site of the transection to fill the cavity. All Sham-operated (Sham) rats underwent a similar operation to those in the SCI group, except that the lower thoracic cord was exposed but not transected. SCI rats received daily assistance in bladder emptying until spontaneous miction recovered.

RES treatment

RES was first dissolved in rapeseed oil and then gently emulsified in a liquid meal (Resource Protein; Nestle, Noisiel, France) in warm water (40°C) for 3 h. Daily RES supplementation lasted 10 days in the treated groups. At 30 min before administration, the mixture was warmed and mixed. Rats were fed RES in a dose equivalent to 400 mg·kg^{-1}·day^{-1} through a 16 G stainless steel curved feeding needle.

Measurement of 25-hydroxyvitamin D [25(OH)D] and calcium (Ca^{2+})

Serum 25(OH)D level was measured using a radioimmune assay (China Institute of Atomic Energy, Beijing, China). Serum concentration of Ca^{2+} was measured using an autoanalyser (Hitachi 7170; Hitachi, Tokyo, Japan).

Measurement of osteocalcin (OCN)

Serum OCN (a specific product of the osteoblast) is a bone formation marker. The concentration of serum osteocalcin was measured using a radioimmunoassay kit (China Institute of Atomic Energy).

Measurement of IL-6

Immediately after decapitation, 2 mL of blood was collected and serum sample was obtained by centrifugation at 1000 rpm for 15 min. Serum IL-6 levels were evaluated using ELISA kits (R&D Systems, Minneapolis, MN, USA).

Measurement of deoxypyridinoline (DPD)

Urinary DPD (the breakdown product of collagen during bone resorption) is a bone resorption marker. DPD excretion was measured using an OSTEOLINKS-DPD EIA kit (Sumitomo Seiyaku, Osaka, Japan), and the data were corrected for the urinary creatinine concentration. Creatinine was quantified with QuantiChrom Creatinine Assay Kits (BioAssay Systems, Hayward, CA, USA).

Assay of malondialdehyde (MDA) levels

The frozen distal femurs were put in a mortar and pestle that contained liquid nitrogen and ground to a fine powder immersed in liquid nitrogen. The frozen powder was transferred into a tube containing 1 × RIPA buffer (Beyotime, Jiangsu, China) supplemented with 1 mM phenylmethylsulphonyl fluoride and protease inhibitor cocktail.

Bone homogenates and plasma were used for the determination of MDA (a presumptive marker of oxidant-mediated lipid peroxidation) using a kit (Cayman, Ann Arbor, MI, USA). MDA content of bone homogenates was calculated as $\mu mol \cdot g^{-1}$ of protein.

Measurement of serum total antioxidant capacity (TAC)

Serum TAC was measured with an Antioxidant Assay Kit (Cayman). The final results were expressed as μmol trolox equivalents$\cdot mL^{-1}$.

Assay of total sulfhydryl (t-SH) levels

Bone homogenates was used for the determination of t-SH using a Glutathione Assay Kit (Cayman). t-SH content of bone homogenates was calculated as $\mu mol \cdot g^{-1}$ of protein. The protein concentration was determined with BSA as a standard by a Bradford assay.

Bone mineral content (BMC) and BMD measurement

BMD and BMC were measured *ex vivo* with a dual energy X-ray absorptiometry NORLAND XR-46 (Norland Co., Fort Atkinson, WI, USA) using the small-animal programme set to a high-resolution mode. Samples were placed on an acrylic platform of uniform 38.1 mm thickness. The BMC of the whole tibiae was obtained. The BMD of proximal tibiae was obtained. The coefficient of variation was 3.0% for BMC and 1% for BMD.

Micro-CT

Trabecular bone morphometry within the metaphyseal region of proximal tibiae was quantified using micro-CT (μCT40, Scanco Medical AG, Zurich, Switzerland, 10.5 μm voxel size, 55 kVp, 145 μA) with a threshold value of 240. The proximal tibial metaphysis was scanned in 250 slices (thickness, 13 μm) in the dorsoventral direction. Three-dimensional reconstruction of bone was performed using the triangulation algorithm. Trabecular morphometry was characterized by measuring the bone volume fraction (bone volume/total volume, BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and trabecular number (Tb.N.).

Bone histomorphometry

The proximal tibiae were incubated with the Villanueva bone stain for 7 days, dehydrated in graded ethanols and xylene, and embedded undecalcified in methyl methacrylate. Frontal sections (4 μm thick) were cut with vertical bed microtomes (Leica, Rockleigh, NJ, USA) and affixed to slides precoated with a 1% gelatin solution. Then they were stained according to the Von Kossa method with a tetrachrome counterstain (Polysciences, Warrington, PA, USA). Histomorphometric data were collected with the Bioquant Bone Morphometry System (R&M Biometrics Corp., Nashville, TN, USA). Eroded surface, osteoclast surface and osteoblast surface were obtained.

Mineral apposition rate (MAR) and bone formation rate (BFR) were analysed by the calcein double-labelling method. Accordingly, mice were intraperitoneally injected twice with 20 $mg \cdot kg^{-1}$ calcein 7 and 2 days, respectively, before killing. The tibiae were kept in 90% ethanol and embedded. Longitudinal sections were made. Dynamic histomorphometric measurements were made using a computer and digitizer tablet (Osteomeasure; Osteometrics Inc., Atlanta, GA, USA) interfaced to a Leitz microscope (Leitz, Wetzlar, Germany) with a drawing tablet. All measurements were done to the metaphyseal region distal to the growth plate region. To estimate bone formation rate, double-labelled and single-labelled areas were traced and calculated as described (Jilka *et al.*, 1996; Weinstein *et al.*, 1997). Terminology used is that recommended by the Histomorphometry Nomenclature Committee of the American Society of Bone and Mineral Research (Parfitt *et al.*, 1987).

Measurement of mechanical properties

Using a mechanical strength analyser (TK-252CC; Muromachi Kikai Co., Ltd, Tokyo, Japan), the mechanical strength of left femur was measured as the method described previously (Mosekilde *et al.*, 1993; Katsumata *et al.*, 1995).

For the three-point bending test, the left femur was placed on a special holding device with supports located 12 mm apart. A bending force was applied with the cross head at a speed of 20 $mm \cdot min^{-1}$, until a fracture occurred. From the load–displacement curve, the ultimate compressive load (N), the stiffness ($N \cdot mm^{-1}$) and the energy (mJ) were obtained.

Quantitative real-time PCR analysis (qRT-PCR)

The frozen distal femurs and tibiae were put in a RNase-free mortar and pestle that contained liquid nitrogen and ground to a fine powder immersed in liquid nitrogen. The frozen powder was transferred into a tube containing Trizol (Life Technologies Inc., Gaithersburg, MD, USA) and total RNA was isolated, according to the manufacturer's protocol. One microgram of total RNA was treated with DNase I (Invitrogen, Camarillo, CA, USA) in a total volume of 10 and 5 μL (500 ng) of this batch was reverse transcribed with 200 U SuperScript II (Invitrogen) using 500 ng oligo-dT and 250 ng random hexamers. RT-PCR analysis was performed with a QuantiTectTM SYBR® Green PCR (Tiangen, Shanghai, China) according to the manufacturer's instructions. The RT-PCR data were based on SYBR green amplification. The sequences of primers are listed in Table 1. The highly specific measurement of mRNA

Table 1
Sequences of oligonucleotides used as primers

Target gene		Sequence (5′-3′)
TRAP	Sense	AATTGCCTACTCCAAGATCTCCAA
	Antisense	GCGGAACTTTGAAACGCAAA
RANKL	Sense	CCATCGGGTTCCCATAAAGTC
	Antisense	CCTGAAGCAAATGTTGGCGTA
OPG	Sense	GCTCCTGGCACCTACCTAAA
	Antisense	GTAGCGCCCTTCCTCACATT
IL-6	Sense	TCCTACCCCAACTTCCAATGCTC
	Antisense	TTGGATGGTCTTGGTCCTTAGCC
OCN	Sense	TCTCTGCTCACTCTGCTGG
	Antisense	GTGGTGCCATAGATGCGCT
PPARγ	Sense	CTTTACCACGGTTGATTTCTCCA
	Antisense	GCAGGCTCTACTTTGATCGCACT
aP2	Sense	AGGAAAGTGGCCGGTATGGC
	Antisense	CCACGCCCAGTTTGAAGGAA
LPL	Sense	TGGCAGGAAGTCTGACCAACAAG
	Antisense	AATCCGCATCATCAGGAGAAAGG
Sost	Sense	GGCAAGCCTTCAAGAATGATGCCA
	Antisense	TGTACTCGGACACGTCTTTGGTGT
Wnt1	Sense	GGGTTTCTGCTACGTTGCTACT
	Antisense	GGAGGTGATTGCGAAGATAAAC
Wnt5a	Sense	TCATGAACTTGCACAACAATGA
	Antisense	CCGTCTTAAACTGGTCATAGCC
Axin2	Sense	GAGCCTGTCAACCCCTACTATG
	Antisense	TCCAACTTTTCTTCAGCCTCTC
Ctnnb1	Sense	AACGGCTTTCGGTTGAGCTG
	Antisense	TGGCGATATCCAAGGGCTTC
Lrp5	Sense	CTGCTGGGGGACTTCATCTACTGGAC
	Antisense	GGGAGGAGTGGAACACCAGGATGTC
IGF-1	Sense	GTGGACCGAGGGGCTTTTACTTC
	Antisense	TTTGCAGCTTCGTTTTCTTGTTTG
IGF-1R	Sense	CTGCGGCGATGAAGAGAAGAAAA
	Antisense	TACCGGTGCCACGTTATGATGATT
IGFBP5	Sense	ACATGGAAGCTTCCCTCCAGG
	Antisense	CGTCACTCAACGTTACTGCTG
GAPDH	Sense	TATCACTCTACCCACGGCAAG
	Antisense	ATACTCAGCACCAGCATCACC

was carried out for tartrate-resistant acid phosphatase (TRAP), RANKL, osteoprotegerin (OPG), osteocalcin (OCN), IL-6, peroxisome proliferator-activated receptor gamma (PPARγ), adipose-specific fatty-acid-binding protein (aP2), lipoprotein lipase (LPL), Sost, Wnt1, Wnt5a, low-density lipoprotein-related protein 5 (Lrp5), insulin-like growth factor 1 (IGF-1), receptor for insulin-like growth factor 1 (IGF-1R), insulin-like growth-factor-binding-protein 5 (IGFBP5), Axin2, ctnnb1 and glyceraldehyde phosphate dehydrogenase (GAPDH) using the LightCycler system (Bio-Rad, Carlsbad, CA, USA). PCR amplification was performed in 96-well optical reaction plates for 40 cycles, with each cycle at 94°C for 30 s, 58–63°C for 30 s, and 72°C for 60 s. Each sample was run and analysed in duplicate. Target mRNA levels were adjusted as the values relative to GAPDH, which was used as the endogenous control to ensure equal starting amounts of cDNA. The fold-change relative to values of sham-operated group were obtained and used to express the experimental change in gene expression.

Study design

SD rats were divided into four groups (n = 41–44 in each group) and treated for 10 days as follows: (i) sham-operated rats (sham); (ii) sham-operated rats received RES (sham + RES); (iii) SCI rats (SCI); and (iv) SCI rats received RES (SCI + RES). 10 days later, femurs and tibiae were removed and the plasma and urine were collected for measurements described as earlier.

Statistical analysis

All the data are presented as mean ± SD. Comparison among groups was analysed using a two-way ANOVA followed by Bonferroni's t-test. $P < 0.05$ was considered statistically significant. Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) 11.0.0 software (SPSS Inc., Chicago, IL, USA).

Results

Effects of treatment with RES on general data in SCI rats

The body mass of SCI rats was lower than that in sham-operated rats. Serum Ca^{2+}, 25(OH)D and osteocalcin of SCI rats were lower than those in sham-operated rats. Urinary DPD was higher than that in sham-operated rats.

Treatment of SCI rats with RES reduced urinary DPD and increased serum osteocalcin significantly, increased body mass, but not significantly. Treatment of SCI rats with RES had no significant effect on serum Ca^{2+} and 25(OH)D (Table 2).

Treatment of sham-operated rats with RES had no significant effect on these parameters.

Effects of treatment with RES on bone geometric and microstructural parameters of SCI rats

As shown in Table 3, SCI in rats resulted in a significant reduction in tibial BMC, BMD, BV/TV, Tb.Th, Tb.N, MAR, BFR/bone surface (BS), and Ob.S/BS and femoral ultimate load, stiffness, and energy, and led to an enhancement in tibial Tb.Sp, ES/BS and Oc.S/BS.

Treatment of SCI rats with RES significantly increased tibial BMC, BMD, BV/TV, Tb.Th, Tb.N, MAR, BFR/BS, and Ob.S/BS and femoral ultimate load, stiffness, and energy, and decreased tibial Tb.Sp, ES/BS, and Oc.S/BS.

Treatment of sham-operated rats with RES had no significant effect on these parameters.

Table 2

Effects of treatment with RES on general data in SCI rats

	Sham	Sham + RES	SCI	SCI + RES
Body mass (g)	235 ± 22	239 ± 27	209 ± 24[a]	218 ± 21
Serum Ca^{2+} ($mmol \cdot L^{-1}$)	2.36 ± 0.17	2.39 ± 0.26	1.87 ± 0.22[a]	1.92 ± 0.23[a]
Serum 25(OH)D ($nmol \cdot L^{-1}$)	142 ± 18	149 ± 20	102 ± 13[a]	105 ± 15[a]
Serum osteocalcin ($ng \cdot mL^{-1}$)	0.125 ± 0.028	0.136 ± 0.021	0.087 ± 0.019	0.132 ± 0.036[b]
DPD ($nM \cdot nM$ creatinine^{-1})	19.6 ± 3.8	17.8 ± 3.2	53.4 ± 8.2[a]	28.8 ± 5.4[a,b]

Values are represented as mean ± SD.
Sham, sham-operated.
n = 10–12 in each group.
[a]$P < 0.05$ versus the sham-operated group.
[b]$P < 0.05$ versus the SCI group.

Table 3

Effects of treatment with RES on bone geometric and microstructural parameters of SCI rats

	Sham	Sham + RES	SCI	SCI + RES
Tibia				
BMC (g)	0.552 ± 0.058	0.591 ± 0.062	0.428 ± 0.051[a]	0.535 ± 0.057[b]
BMD ($g \cdot cm^{-2}$)	0.195 ± 0.022	0.204 ± 0.029	0.151 ± 0.018[a]	0.182 ± 0.025[b]
BV/TV (%)	16.3 ± 3.1	17.0 ± 3.6	7.9 ± 2.2[a]	12.5 ± 2.8[a,b]
Tb.Th (μm)	80.3 ± 11.5	82.1 ± 12.7	56.2 ± 8.8[a]	72.2 ± 10.2[b]
Tb.Sp (μm)	562.7 ± 80.3	574.2 ± 86.2	962.1 ± 102.5[a]	660.5 ± 90.4[a,b]
Tb.N ($n \cdot mm^{-1}$)	4.2 ± 1.4	4.3 ± 0.9	2.3 ± 0.7[a]	3.5 ± 0.8[b]
MAR ($\mu m \cdot day^{-1}$)	1.71 ± 0.19	1.79 ± 0.17	1.28 ± 0.20[a]	1.66 ± 0.26[b]
BFR/BS ($\mu m^3 \cdot \mu m^{-2} \cdot day^{-1}$)	0.57 ± 0.07	0.60 ± 0.06	0.40 ± 0.08[a]	0.54 ± 0.09[b]
ES/BS (%)	6.29 ± 0.65	6.01 ± 0.53	12.88 ± 1.32[a]	8.55 ± 0.90[a,b]
Ob.S/BS (%)	3.56 ± 0.34	3.69 ± 0.37	1.88 ± 0.21[a]	3.20 ± 0.37[b]
Oc.S/BS (%)	2.82 ± 0.45	2.75 ± 0.41	7.06 ± 0.94[a]	4.24 ± 0.66[a,b]
Femur				
Ultimate load (N)	209 ± 47	214 ± 42	141 ± 30[a]	182 ± 39[b]
Stiffness ($N \cdot mm^{-1}$)	480 ± 96	491 ± 81	358 ± 74[a]	457 ± 85[b]
Energy (mJ)	78 ± 16	80 ± 14	50 ± 13[a]	67 ± 15[b]

Values are represented as mean ± SD.
Sham, sham-operated.
n = 10–12 in each group.
[a]$P < 0.05$ versus the sham-operated group.
[b]$P < 0.05$ versus the SCI group.

Effect of treatment with RES on the biomarkers of osteoblastogenesis and osteoclastogenesis in femurs of SCI rats

Compared with sham-operated rats, mRNA levels of TRAP (Figure 1A) and ratio of RANKL-to-OPG (Figure 1B) were higher in femurs of SCI rats; and mRNA levels of osteocalcin (Figure 1C) was lower in femurs of SCI rats. Treatment of SCI rats with RES decreased TRAP mRNA levels, reduced ratio of RANKL-to-OPG, and increased osteocalcin mRNA levels in femurs.

Effect of treatment with RES on oxidative stress and inflammation in femurs of SCI rats

When compared with sham-operated rats, serum total capacity of antioxidant (TCA) (Figure 2A) was lower, plasma and femoral MDA content (Figure 2B, C) were higher, and femoral t-SH content (Figure 2D) was lower in SCI rats. Treatment of SCI rats with RES-enhanced serum TCA and femoral t-SH, and reduced plasma and femoral MDA content, which indicated that

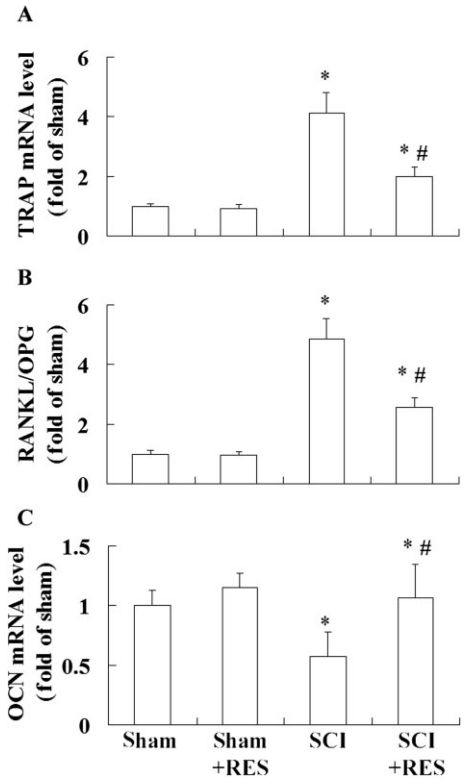

Figure 1

Effect of treatment with RES on the biomarkers of osteoblastogenesis and osteoclastogenesis in femurs of SCI rats. Sham-operated rats and SCI rats were treated with RES for 10 days. Femurs were removed for determination of mRNA levels of TRAP, RANKL, OPG and OCN by qRT-PCR method. The ratio RANKL to OPG was obtained (B). The mRNA levels of TRAP (A) and osteocalcin (C) were adjusted as the values relative to GAPDH. The fold-change relative to values of sham-operated group were obtained and used to express the experimental change in gene expression. n = 10–12 in each group; Values are represented as mean ± SD. $^{*}P < 0.05$ versus the sham-operated group; $^{\#}P < 0.05$ versus the SCI group.

treatment of SCI rats with RES abated oxidative stress in femurs.

SCI in rats enhanced serum IL-6 levels (Figure 2E) and femoral IL-6 mRNA levels (Figure 2F). Treatment of SCI rats with RES reduced serum IL-6 levels and femoral IL-6 mRNA levels, indicating that treatment of SCI rats with RES suppressed inflammation in femurs.

Effect of treatment with RES on genes expression of PPARγ signalling pathway in femurs of SCI rats

When compared with sham-operated rats, femoral mRNA levels of PPARγ (Figure 3A), aP2 (Figure 3B) and LPL (Figure 3C) were higher in SCI rats. Treatment of SCI rats with RES reduced femoral mRNA levels of PPARγ, aP2 and LPL, which indicated that treatment of SCI rats with RES suppressed PPARγ signalling in femurs.

Similar results were also found in tibiae (Supporting Information Figure S1A–C).

Effect of treatment with RES on genes expression of Wnt/β-catenin signalling pathway in femurs of SCI rats

When compared with sham-operated rats, Sost mRNA level (Figure 4A) was higher, and Wnt1 (Figure 4B), Lrp5 (Figure 4D), Axin2 (Figure 4E) and ctnnb1 (Figure 4F) mRNA levels were lower in femurs of SCI rats. Treatment of SCI rats with RES enhanced femoral mRNA levels of Wnt1, Lrp5, Axin2 and ctnnb1, which indicated that treatment of SCI rats with RES restored Wnt/β-catenin signalling pathway in femurs. Treatment of SCI rats with RES had no significant effect on femoral Sost mRNA level. Femoral Wnt5a mRNA (Figure 4C) level was similar among four groups.

Similar results were also found in tibiae (Supporting Information Figure S1D–I).

Effect of treatment with RES on genes expression of IGF-1 signalling pathway in femurs of SCI rats

When compared with sham-operated rats, femoral mRNA levels of IGF-1 (Figure 5A), IGF-1R (Figure 5B), and IGFBP5 (Figure 5C) were lower in SCI rats. Treatment of SCI rats with RES-enhanced femoral mRNA levels of IGF-1 and IGF-1R, which indicated that treatment with RES restored IGF-1 signalling in femurs of SCI rats. Treatment of SCI rats with RES had no significant effect on femoral IGFBP5 mRNA level.

Similar results were also found in tibiae (Figure S1J–L).

Discussion

In this study, we presented a novel application of RES in attenuating bone loss induced by SCI in rats for the first time. More specifically, RES treatment was associated with a significant reduction in bone resorption and a significant enhancement of bone formation. Increased osteoclast formation and bone resorption after SCI has been demonstrated in rodents and humans (Pietschmann *et al.*, 1992; Morse *et al.*, 2008; 2011). Findings with respect to SCI-mediated effects on osteoblasts are less consistent. Jiang *et al.* (2007) and Liu *et al.* (2008) found that bone formation rate in the secondary spongiosae of tibial metaphysis was higher than that in sham-operated groups 3 or 4 weeks after SCI. Morse *et al.* found that bone formation rate at the distal femoral metaphysis was lower than that in sham-operated groups at 10 days after SCI (Morse *et al.*, 2008) and osteoblast number at the distal femur reduced to 34% at 5 days after injury (Morse *et al.*, 2011). In this study, at 10 days after SCI osteoblast, number and bone formation rate were reduced. These results indicated that osteoblastic function might be impaired at the early stage of SCI, and restored and even enhanced at the late stage of SCI.

Vitamin D deficiency was an important risk factor contributing to declining bone mass and increased fracture risk post-SCI (Hummel *et al.*, 2012). Treatment with RES had no effect on serum 25(OH)D level, which indicated that the beneficial effect of RES on osteoporosis was Vitamin D-independent.

Oxidative stress was considered as a hallmark of injury of SCI (Jia *et al.*, 2012). In this study, SCI in rats led to an

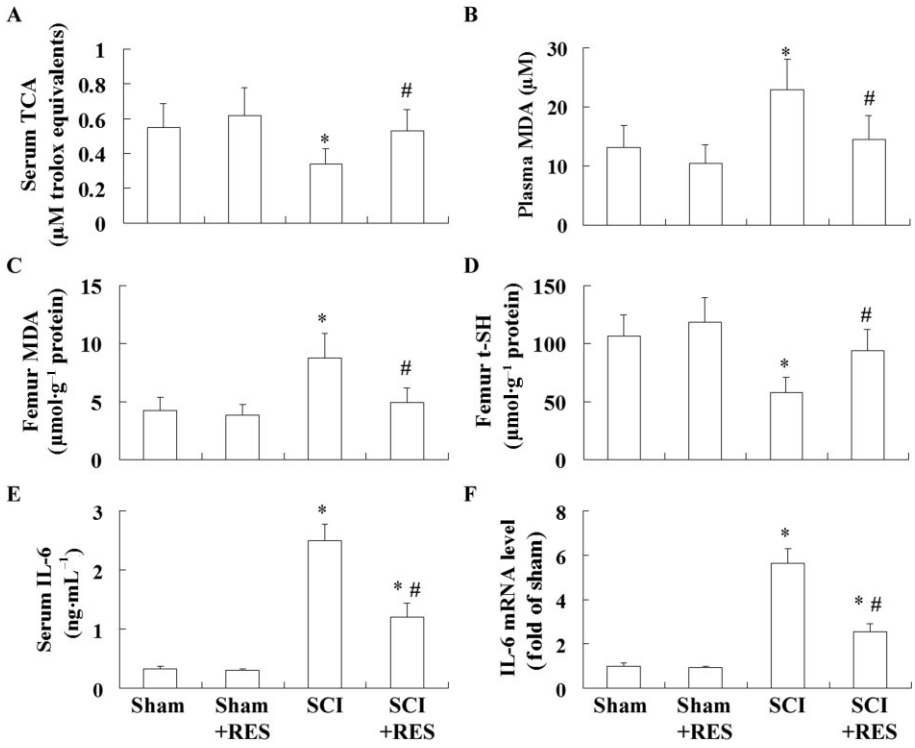

Figure 2

Effect of treatment with RES on oxidative stress and inflammation in femurs of SCI rats. Sham-operated rats and SCI rats were treated with RES for 10 days. Serum TCA (A), plasma and femoral MDA (B, C), femoral t-SH (D), and serum and femoral IL-6 (D, E) were measured. n = 10–12 in each group; Values are represented as mean ± SD. *P < 0.05 versus the sham-operated group; $^{\#}P$ < 0.05 versus the SCI group.

enhancement of serum MDA and femoral MDA, indicating that oxidative stress might be involved in the development of osteoporosis after SCI. In addition, a report from Sun *et al*. revealed that oxidative stress might play an important role in the osteoporosis induced by hindlimb suspension-a model of disuse (Sun *et al*., 2013). In this work, treatment with RES abated oxidative stress in femurs, indicating that the antioxidant effect of RES might contribute to its anti-osteoporotic effect.

Apart from oxidative stress, inflammation might be another factor involved in the development of bone loss after SCI. Demulder *et al*. reported that levels of IL-6 was increased in samples from the SCI patients (Demulder *et al*., 1998), which might promote osteoclast recruitment from marrow precursors and enhance osteoclast activity in sublesional bones. A recent report revealed that RES treatment exerted neuroprotective effect in SCI rats through suppressing inflammation (Liu *et al*., 2011). In this work, RES treatment reduced serum and femoral IL-6 levels in SCI rats, indicating that anti-osteoporotic effect of RES might be contributed to its suppression on inflammation in sublesional bones of SCI rats.

Our work revealed up-regulated PPARγ signalling in the femurs from SCI rats, which was consistent with a recent report (Yan *et al*., 2012). PPARγ in osteoblasts suppressed the mature osteoblast phenotype and induced genes associated with an adipocyte-like phenotype, such as aP2, fatty acid synthase and LPL (Lecka-Czernik *et al*., 1999). In addition, targeted PPARγ deletion could impair osteoclastic differentiation and bone resorption, resulting in osteoporosis (Wan *et al*., 2007). In this study, RES treatment suppressed PPARγ signalling in the femurs from SCI rats.

Our work revealed reduced Wnt/β-catenin signalling in the femurs from SCI rats, which was consistent with recent reports (Jiang *et al*., 2011; Yan *et al*., 2012). Wnt/β-catenin promoted bone formation and suppressed bone resorption in postnatal growing mice (Chen and Long, 2013). The reduced Wnt/β-catenin signalling pathway in the femurs from SCI rats might result from up-regulated expression of Sost. Sclerostin, the protein product of the Sost gene, is a potent inhibitor of bone formation. Sclerostin mediated bone response to mechanical unloading through antagonizing Wnt/beta-catenin signalling (Robling *et al*., 2008; Tu *et al*., 2012). In this study, RES treatment had no effect on Sost mRNA levels, but restored Wnt/beta-catenin signalling. It was reported that increased lipid oxidation in osteoblast caused oxidative stress, which in turn increased PPARγ expression, and diminished Wnt signalling in the skeleton (Almeida *et al*., 2009). Therefore, the effect of RES on PPARγ and Wnt/β-catenin signalling might be secondary to its antioxidant effect.

Our work revealed reduced IGF-1 signalling in the femurs from SCI rats. It was reported that the average plasma IGF-1 level was significantly lower in SCI patients and patients with tetraplegia (Shetty *et al*., 1993; Bauman *et al*., 1994). Jiang *et al*. also found depressed IGF-1 signalling in the tibiae from SCI rats (Jiang *et al*., 2011). IGF-I has been shown to be a

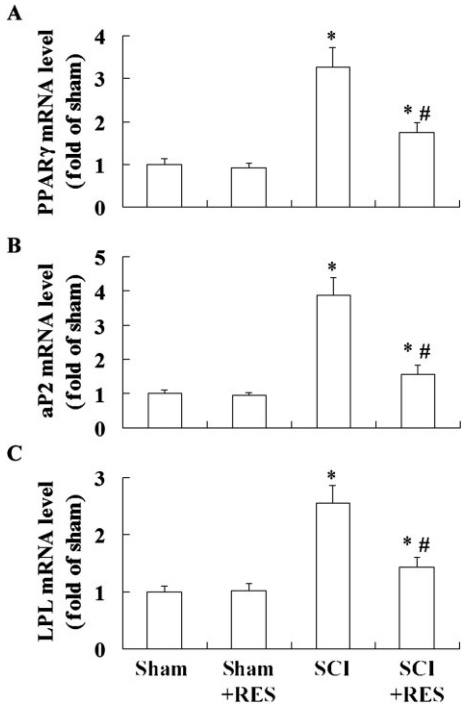

Figure 3

Effect of treatment with RES on genes expression of PPARγ signalling in femurs of SCI rats. Sham-operated rats and SCI rats were treated with RES for 10 days. PPARγ (A), aP2 (B) and LPL (C) mRNA levels were determined by qRT-PCR method. n = 10–12 in each group; Values are represented as mean ± SD. *P < 0.05 versus the sham-operated group; $^{\#}P$ < 0.05 versus the SCI group.

potent neurotrophic factor that promotes the growth of projection neurons, dendritic arborization and synaptogenesis, and has been demonstrated neuroprotective effect for SCI rats (Hung *et al.*, 2007). IGF-1 is the most abundant growth factor deposited in the bone matrix and has been implicated in the coupling process through its actions on MSC differentiation (Hayden *et al.*, 1995). IGF-1 released from bone matrix stimulated osteoblastic differentiation of MSCs by activation of mammalian target of rapamycin during bone remodelling (Xian *et al.*, 2012). Suzue *et al.* (2006) demonstrated that suppressed IGF-I signalling might contribute to decreased bone formation during denervation, and denervation of the sublesional bones also occurred after complete SCI. Therefore, suppressed IGF-I signalling might contribute to decreased bone formation after SCI in rats. In this RES treatment restored the IGF-I signalling in the femurs from SCI rats.

A limitation of our work should be noted. Although unloading is an important factor in the pathogenesis of osteoporosis after SCI, neural lesion also seems to be involved in this process (Jiang *et al.*, 2006). RES significantly promoted the recovery of neuronal function after SCI (Yang and Piao, 2003; Liu *et al.*, 2011). Whether improvement of RES on bone loss was due to direct effect of RES on bone or indirect due to positive effect of RES after SCI or both was elusive, which required further investigation.

In the present study, the dose of RES was high, but was reported to be well tolerated by animals (Lagouge *et al.*, 2006), and recent data failed to show a toxic effect of RES in rats even at doses reaching 700 mg·kg^{-1}·day^{-1} (Williams *et al.*, 2009). The dose of 400 mg·kg^{-1} was based on previous studies in which doses ranging from 10 to 400 mg·kg^{-1} displayed positive effects on adiposity and insulin sensitivity (Szkudelska and Szkudelski, 2010). Very few adverse effects have been documented due to the low bioavailability of RES and no side effects were seen in this study. However, the dose used in our work was not applicable in clinical trials, which

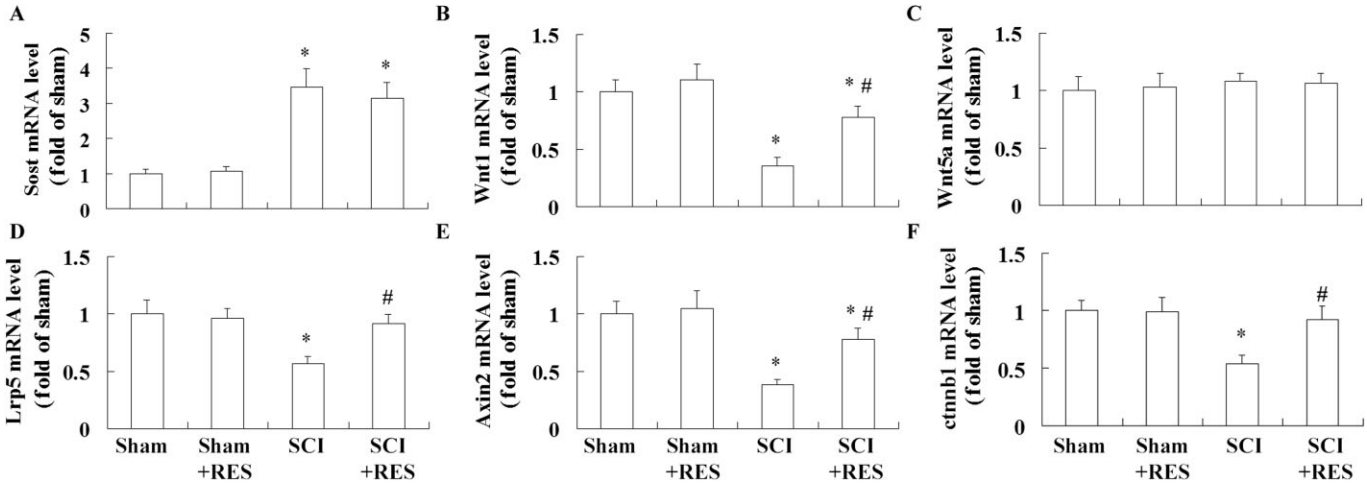

Figure 4

Effect of treatment with RES on genes expression of Wnt/β-catenin signalling in femurs of SCI rats. Sham-operated rats and SCI rats were treated with RES for 10 days. Sost (A), Wnt1 (B), Wnt5a (C), Lrp5 (D), Axin2 (E) and ctnnb1 (F) mRNA levels were determined by qRT-PCR method. SCI, spinal cord injury; Lrp5, low-density lipoprotein-related protein5; n = 10–12 in each group. Values are represented as mean ± SD. *P < 0.05 versus the sham-operated group; $^{\#}P$ < 0.05 versus the SCI group.

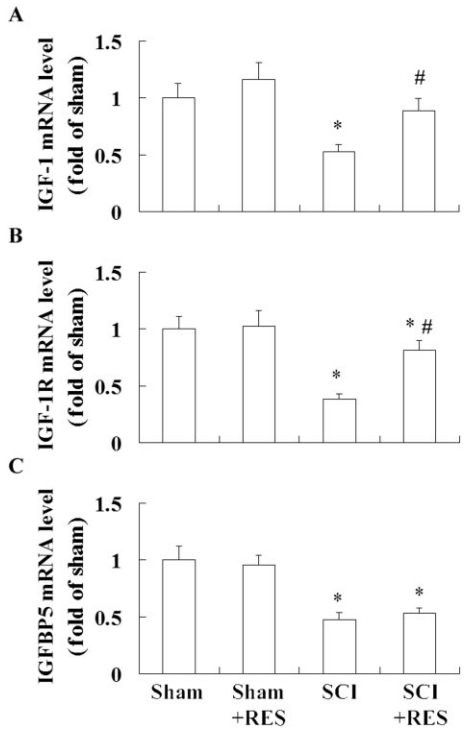

Figure 5

Effect of treatment with RES on genes expression of IGF-1 signalling in femurs of SCI rats. Sham-operated rats and SCI rats were treated with RES for 10 days. IGF-1 (A), IGF-1R (B) and IGFBP5 (C) mRNA levels were determined by qRT-PCR method. SCI, spinal cord injury; IGF-1, insulin-like growth factor 1; IGF-1R, receptor for insulin-like growth factor; IGFBP5, insulin-like growth-factor-binding-protein 5; $n = 10–12$ in each group. Values are represented as mean ± SD. $^{*}P < 0.05$ versus the sham-operated group; $^{\#}P < 0.05$ versus the SCI group.

might be another limitation of our work. Some additional works about lower dose testing and further clinical trials were required.

In conclusion, treatment with RES attenuated sublesional bone loss in SCI rats, associated with abating oxidative stress, attenuating inflammation, depressing PPARγ signalling, and restoring Wnt/β-catenin and IGF-1 signalling. RES could thus be envisaged as a nutritional countermeasure for osteoporosis after SCI but remains to be tested in humans.

Conflicts of interest

None to declare.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

http://dx.doi.org/10.1111/bph.12301

Figure S1 Effect of treatment with RES on genes expression of pathways about PPARγ signalling, Wnt/β-catenin signalling, and IGF-1 signalling in tibiae of SCI rats. Sham-operated rats and SCI rats were treated with RES for 10 days. PPARγ (A), aP2 (B), LPL (C), Sost (D), Wnt1 (E), Wnt5a (F), Lrp5 (G), Axin2 (H), ctnnb1 (I), IGF-1 (J), IGF-1R (K) and IGFBP5 (L) mRNA levels were determined by qRT-PCR method. SCI, spinal cord injury; PPARγ, peroxisome proliferator-activated receptor gamma; aP2, adipose-specific fatty-acid-binding protein; LPL, lipoprotein lipase; Lrp5, low-density lipoprotein-related protein5; IGF-1, insulin-like growth factor 1; IGF-1R, receptor for insulin-like growth factor; IGFBP5, insulin-like growth-factor-binding-protein 5; n = 10–12 in each group; Values are represented as mean ± SD. *$P < 0.05$ versus the sham-operated group; #$P < 0.05$ versus the SCI group.