CHEMICAL ENGINEERING TRANSACTIONS
VOL. 46, 2015
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Peiyu Ren, Yancang Li, Huiping Song

ISBN 978-88-95608-37-2; **ISSN** 2283-9216
DOI: 10.3303/CET1546012

Prevention of Radix Rehmanniae Preparata on Glucocorticoid-Induced Osteoporosis in Rats

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The aim of present study is investigating the preventive effects of Radix Rehmanniae Preparata (PPR) on glucocoroticoids-induced osteoporosis rats. Forty-eight 3-month SD rats were divided into baseline (BAS) group, age control (CON) group, and glucocorticoids group. The glucocorticoids group were further divided into GC group (n=8) treated with glucocorticoids only, GC+ CaD group was given glucocorticoids plus calcium carbonate (375 $mg \cdot kg^{-1} \cdot d^{-1}$ oral) and vitamin D_3 50 U/kg per day oral; GC+ low dose RRP group were treated with glucocoroticoids plus PPR (4 $ml \cdot kg^{-1} \cdot d^{-1}$ oral); GC+ high dose RRP group were treated with glucocoroticoids plus PPR (7.5 $ml \cdot kg^{-1} \cdot d^{-1}$oral). After 24 weeks of treatment, the rats were sacrificed under sterile conditions. The right tibiae were removed for bone histomorphometry, and the right femurs were stored for bone biomechanics testing. In GC group, body weight, Alkaline phosphatase (ALP), C-terminal propeptide of type I procollagen (PICP), were increased.% Tb.Ar, Tb.Wi, Tb.N and modulus of rigidity indices decrease significantly and Tb.Sp indices increased significantly in GC group compared with the CON group, while RRP and calcium carbonate with vitamin D_3 can prevented the decreased in bone histomorphometry and biomechanical quality. The present results suggest that RRP prevent glucocorticoid-induced osteoporosis by improving bone formation.

1. Introduction

Glucocorticoids (GCs) have been widely used in the treatment for inflammatory and autoimmune disease such as inflammatory bowel disease, allergy and hypersensitivity disorders, et al. However, pharmacological dosages of GCs often lead to glucocoroticoids-induced osteoporosis (GIO) (Nishimura, J. er al (2000), Clowes, J.et al. (2001)). GIO is well recognized as a risk factor of fracture risk in osteoporosis. Due to the side effects of GC, GC often have to be tolerated during the treatment, which limit the long-term administration (Shah SK and Gecys GT (2006)). An estimated 1.5 million postmenopausal women and men aged ≥50 years were using an oral glucocorticoid at any time between 2005 and 2010,however, only 28.4% of postmenopausal women and 9.7% of men aged ≥50 reported use of an antiosteoporosis pharmaceutical (Robert A et al (2014)). Therefore, GIO represent a major problem, and development of drugs to prevent GIO is worth noting. Now the therapeutic measure of GIO includes calcium, vitamin D, bisphosphonates, raloxifene, PTH, hormone replacement and calcitonin which are similar to the treatment of post-menopausal osteoporosis. Due to the impairment in calcium absorption mediated by glucocorticoids, calcium and vitamin D are widely used in prevention and treatment for GIO (Iwamoto J et al. (2005)), but it may lead to hypercalcaemia and hypercalciuria in the early treatment. Bisphosphonates prevent bone resorption, play an important role in treatment of GIO , but more and more bisphosphonate complications will happen because of the low turnover state in GIO users with prolonged using of bisphosphonates. Teriparatide, a recombinant human PTH 1–34, is approved internationally for use in GIO. PTH treatment can prevent the bone mass reduction, lessen and perhaps even overcome the side effect of glucocorticoid associated with structural changes in trabecular and cortical bone.In spite of market effect, the expensive cost become heavy burden, The incremental cost per quality-adjusted life year (QUALY) to prevent an incident vertebral fracture was nearly 21,000 euros (Murphy,

Please cite this article as: Wu T.X., Zou L., He H.H., Wu K.F., 2015, Prevention of radix rehmanniae preparata on glucocorticoid-induced osteoporosis in rats, Chemical Engineering Transactions, 46, 67-72 DOI:10.3303/CET1546012

D.R et al. (2012)). As discussed above, current measurement has its merits and defects, the aim of current study is developing new drugs to prevent GIO.

Radix Rehmanniae Preparata (RR) is a traditional Chinese medicinal herb and noted in traditional Chinese medicine (TCM) as a drug (Zhang, R.X et al. (2004)), they are widely studied for their chemical properties and biological activities in food and pharmaceutical industry. Polysaccharide of *Radix Rehmanniae* Preparata (RRP) was a heterosaccharide with a molecular mass of 3.5×10^4 Da. The four monosaccharides, glucose, galactose, fructose and stachyose were identified in the hydrolysate of RRP, and their mol ratio was 0.71:1.21:1.35:1.56 (Chen, C.F et al. (2008)). In addition, recent study showed that the RR prevent osteoporosis in ovariectomized rats, RR could increase the bone density, promote bone formation and improve bone quality (Li Ou et al. (2013)). However, little date has been done on the effect of RR on bone histomorphometry in GIO. Therefore, we need to provide more documents about the prevent effects as well as the underlying mechanisms of RR in GIO.

2. Materials and methods

2.1 Animals

All animal experimentation was conducted in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals of Guangdong Laboratory Animal Monitoring Institute under by National Laboratory Animal Monitoring Institute of China. ALL the Sprague-Dawley rats were acclimatized for 2 weeks to the local housing conditions (temperature 24–26°C, humidity 67%) and were allowed free access to water and normal diets. All rats received subcutaneous injection of tetracycline (20 mg/kg, Sigma ChemicalCo. St. Louis, MO) on days 13 and 14, and calcein (10 mg/kg, Sigma Chemical Co. St. Louis, MO) on 3 and 4 days before sacrifice.

2.2 Experimental protocols

Forty-eight 3-month SD rats,male and female,were randomly divided into 6 groups with 8 rats per group. Above random grouping were carried on by SPSS. The groups were: 1) base group (BAS, the rats were euthanized before the experiment); 2) age control group (CON, was given physiological saline by intraperitoneal injection 75 mg/kg); 3) GC group (GC), was given methylprednisolone (Pfizer manufacturing Belgium NV, Production lot: Y05009) by intraperitoneal injection 75 mg/kg); 4) GC+ CaD group was given methylprednisolone by intraperitoneal injection, plus calcium carbonate (375 $mg\cdot kg^{-1}\cdot d^{-1}$ oral) and vitamin D_3 50 U/kg per day oral; 5) GC+ low dose RRP group was given methylprednisolone by intraperitoneal injection, plus RRP (at a dose of 4 $ml\cdot kg^{-1}\cdot d^{-1}$ oral); 6) GC+high dose RRP group was given methylprednisolone by intraperitoneal injection, plus RRP (at a dose of 7.5 ml·kg-1·d-1 oral).Methylprednisolone was given for 3 days, stop for a week, intraperitoneal injection once a week according to the plan. After 24 weeks of treatment, at the end point, the rats were sacrificed under sterile conditions. Soft tissues were removed and weighed. The right tibiae were removed for bone histomorphometry, and the right femurs were stored at −20°C until further mechanical testing was performed.

2.3 Serum markers assay

Alkaline phosphatase (ALP), serum calcium (Ca), serum phosphorus (P), C-terminal propeptide of type I procollagen (PICP), C terminal propeptide of type I collagen (CICP), and deoxypyridinoline (DPD) were measured with assay kits.

2.4 Bone histomorphometry

The right tibial were opened to expose the bone marrow cavity using an Isomet Low Speed Saw(Buehler, Lake Bluff, Illinois, USA) and fixed in 10% phosphatebuffered formalin for 24 hours. They were then dehydrated ingraded ethanol, defatted in xylene, and embedded undecalcified in methyl methacrylate [11]. Frontal sections were cut at thicknesses of 4-and 8-mm. The 4-mm sections were stained by toluidin blue,for static histomorphometric measurements. The 8-mm thick sections were used for dynamic histomorphometric an alyses (Cui L et al. (2004)). Static measurements included total tissue volume (TV), trabecular bone volume (BV), marrow fatty area (F. Ar), trabecularbone surface (BS). These parameters were used to calculate Trabecul*ar ar*ea (%Tb.Ar), Trabecular width (Tb.Wi), Trabecular number (Tb.N) and Trabecular separation (Tb.Sp) All histomorphometric arameters were in accordance with the published studies. This is the key to determine whether the model is successful.

2.5 Bone biomechanics testing

The right femurs were moisturized by soaking them in saline solution with the residual muscle removed. Biomechanical quality of the right femur was determined by carrying out a three-point bending test using a material testing machine (MTS). The right femur was placed horizontally on two supports separated by a distance of 20 mm and equidistant from the ends. Central loading was then applied at a constant deformation rate of 2mm/min, and the load and displacement were recorded until the bone was broken. From the load-

deformation curve, maximum load (the maximum force the bone can resist, N) and stiffness (load-displacement curve slope, N/mm) were obtained.

2.6 Radix rehmanniae preparata preparation

A native polysaccharideswas isolated from Radix Rehmanniae Preparata (RRP) by microwave-assisted extraction (MAE). In the method, the optimized conditions were: the ratio of raw material to water was 1:50 (g/ml); microwave irradiation power was 550W; extraction temperature was 60℃; extraction time was 30min. As a result, the average yield of RRP using MAE was 9.14%, and R.S.D. was 2.63%.

3. Statistic analysis

All date were analyzed using a statistical software (SPSS version 13.0 for Windows; SPSS), they were presented as mean±SD. The statistical differences among groups were evaluated using variance (ANOVA) with Fisher's PLSD test. Skew distribution used firstly Kruskal-Wallis and then Conover's t to compare the differences. A value of $p < 0.05$ was considered statistically significant.

4. Results

4.1 Body weight

All rats were weighed every week. As shown in Tab 1, compared with the control group (CON), body weight decreased significantly in GC group after 24 weeks. In the groups treated with glucocorticoid plus RRP (4 or 7.5 $ml \cdot kg^{-1} \cdot d^{-1}$ oral), body weights increased significantly compared with the GC group.

Table 1: Effect of RRP on the body weight in GIO rats (g)

Group	0week	8week	12week	16week	24week
BAS	184.6±20.0	------	-----	------	------
CON	179.1±14.9	265.5±28.6	312.6±29.7	360.8±11.1	385.0±6.5
GC	181.9±11.8	247.8±21.1	280.9±30.0	299.6±41.2	324.6±54.0 ▲
GC+CaD	189.3±22.4	251.8±17.7	296.2±26.7	327.4±36.9	373.3±33.1 ##
GC+Low dose RRP	185.7±26.5	255.1±21.3	322.0±36.9	347.9±41.4	366.9±26.6 ##
GC+High dose RRP	187.2±18.3	263.8±15.1	296.8±10.0	342.9±27.5	380.0±23.3 ##

▲:*vs* CON, p<0.05; ▲▲:*vs* CON, p<0.01 #:*vs* GC, p<0.05; ##: *vs* GC, p<0.01
•: *vs* GC+ CaD, *p*<0.05 ★: *vs* GC+Low dose RRP, *p*<0.05

4.2 Serum marker assay

As illustrated in Tab 2, compared with CON group, the serum levels of ALP decreased significantly in GC group and in GCLR group (P<0.05). Compared with GC and CON group, the serum levels of Ca in GC+CaD group increased significantly (P<0.05). The serum levels of ALP in GC+CaD group and GCHR group increased significantly (P<0.05) compared with GC group. The serm levels of PCIP lower in GC than in CON, GC+CaD group, GCLR and GCHR group (P<0.05).

Table 2: Effect of RRP on Serum marker assay in GIO rats (Mean±SD)

Group	ALP(U/L)	Ca (mmol/L)	P (mmol/L)	PICP (ng/L)	CICP (ng/L)	DPD
BAS	------	------	-----	------	------	------
CON	98.9±40.2	2.7±0.2	1.3±0.3	299.6±41.2	31.0±1.37	731.6±32.3
GC	62.2±14.0▲	2.1±0.1	1.6±0.4	154.2±6.1▲# ⋆	32.3±1.5	738.6±22.5
GC+CaD	96.5±25.5#	3.6±0.1▲#	1.5±0.1	327.4±36.9#	38.4±2.6	709.3±21.8
GC+Low dose RRP(GCLR)	43.3±20.2▲#•	2.3±0.8	1.7±0.1	347.9±41.4#	31.6±1.9	736.1±19.7
GC+High dose RRP(GCHR)	103.8±38.4#⋆	2.7±0.1	1.7±0.4	342.9±27.5#	31.3±1.7	729.7±42.6

▲:*vs* CON, p<0.05 #:*vs* GC, p<0.05 •: *vs* GC+ CaD, *p*<0.05 ⋆: *vs* GC+Low dose RRP, *p*<0.05

4.3 Bone histomorphometry of cancellous bone

Static histomorphometric measurements of right tibiae were illustrated in Table 3, there was no significance difference in static parameters (%Tb.Ar, Tb.Wi, Tb.N, Tb.Sp) between base group (BAS) and control group (CON). %Tb.Ar, Tb.Wi and Tb.N, indices decrease significantly and Tb.Sp indices increased significantly in GC group compared with the CON.Compared with GC group, %Tb.Ar, Tb.Wi and Tb.N, indices increase significantly and Tb.Sp indices decreased significantly in GC+CaD group (P<0.05). In the groups treated with glucocorticoid plus RRP (4 or7.5 ml·kg^{-1}·d^{-1} oral), %Tb.Ar increased significantly compared with the GC group while Tb.Sp decreased significantly (P<0.05).

Table 3: Effect of RRP on Static parameters of right tibial in GIO rat s(Mean±SD)

Group	**%Tb.ar(%)**	**Tb.Wi(um)**	**Tb.N(#/mm)**	**Tb.Sp(um)**
BAS	25.1±10.1	52.6±16.4	4.2±1.0	110.2±16.8
CON	48.2±5.0	68.4±18.5	18.5±9.9	71.8±12.1
GC	30.8±8.3▲	45.2±11.0▲	7.2±2.6▲	293.5±51.8▲
GC+CaD	40.0±2.1#	66.6±27.8#	16.3±10.1#	97.6±28.7##
GC+Low dose RRP	42.4±13.5#	55.8±1.4	12.0±7.3	100.5±6.9##
GC+High dose RRP	45.2±7.2#	67.0±9.1	13.3±4.9	88.2.9±14.6##

▲:*vs* CON, p<0.05; ▲▲:*vs* CON, p<0.01 #:*vs* GC, p<0.05; ##: *vs* GC, p<0.01
•: *vs* GC+ CaD, *p*<0.05 ⋆: *vs* GC+Low dose RRP, *p*<0.05

4.4 Bone biomechanics parameters

As shown in Tab4, There was no significance difference in bone biomechanics parameters (Load, Rigidity, Breaking strength) in all groups. Compared with CON group, modulus of rigidity in GC group decreased significantly (P<0.05), while modulus of rigidity in groups treated with glucocorticoid plus CaD or RRP (4 or 7.5 ml·kg^{-1}·d^{-1} oral) increased significantly (P<0.05) compared with GC group.

Table 4: Effect of RRP on bone biomechanics parameters in rats lumbar vertebra(Mean±SD)

Group(n)	Load(N)	Rigidity(N/mm)	Breaking strength($N \cdot mm^{-2}$)	modulus of rigidity(MPa)
BAS(8)	296.32±1.8	446.0±68.7	40.18.±2.3	587.7±17.2
CON (10)	332.58±86.2	538.2±30.5	49.85±0.6	630.0±33.1
GC(10)	320.9±44.2	528.7±28.8	42.5±7.7	510.3.3±18.8▲
GC+CaD	345.7±18.3	540.6±27.8	51.3±6.8	617.9±8.1#
GC+Low dose RRP (GCLR)	342.7±13.6	544.8±10.5	50.1±23.1	628.3±16.4#
GC+High dose RRP (GCHR)	360.0±33.9	558.3±37.6	62.8±27.4	645.8±47.1#

▲:*vs* CON, $p<0.05$ #:*vs* GC, $p<0.05$

5. Discussions

Glucocorticoids therapy is strongly limited since extended glucocorticoids can cause serious side effects, glucocoroticoids-induced osteoporosis (GIO) is the common form of secondary osteoporosis. Both advantage and disadvantage exit in current treatment of GIO. In our previous study, RRP can stimulate the proliferation and differentiation of osteoblasts in vitro (Tianxiu Wu et al (2013)). Therefore our present study intends to prove the potential prevention of RRP in GIO. At present many methods are used to make GIO animal models with differences in the dosage and usage method of incretion. The modelling methods in this paper were chosen after a long period grope, because it owns high success rate and low death rate (Xiaohui Sun, 2015 et al). Considering this paper mainly discusses the preventive effect of PPR on GIO, we also made drug intervention on PPR while making the model.

Many studies in vivo (Daohua Xu et al. (2010)) showed that body weight decreased in glucocorticoid-treated rats. The present study display that the body weight in GC decrease significantly, which were consistent with those in previous reports. Excessive glucocorticoid can promote catabolic effect in vivo, including carbohydrate, protein, and fat metabolism. In the groups treated with glucocorticoid plus RRP (4 or 7.5 $ml \cdot kg^{-1} \cdot d^{-1}$ oral), body weights increased significantly compared with the GC group. These results suggest that the protective effects of RRP in glucocorticoid-treated rats (Tian lihua, (2013)).

As shown in Tab 2, the serum levels of ALP in GC and GCLR group decreased significantly than in CON group with no significant difference observed in serum levels of Ca, P and CICP among the above groups. Compared with GC group, the serum levels of ALP in GC+CaD group and GCHR group increased significantly. The results aboved indicated osteoblast activity was inhibited by GC, and high dose RRP can promote osteoblast activity. The serm levels of PCIP reflect the activities of osteoblasts, it is specific index of I type collagen. Treated with GaD or RRP (4 or 7.5 $ml \cdot kg^{-1} \cdot d^{-1}$ oral) increased the serm levels of PCIP in GIO rats, and it was proved again that PPR promote osteoblast activity. There was no significant difference among all groups in the level of CICP.CICP is a specific index which reflects bone resorption. Therefore, the present results can not prove that RRP is related to bone resorption.

The traditional bone histomorphometry is one of the gold standards of the quantitative analysis of bone microarchitecture (Jie Cai et al. (2015)). Our study established a rat model of glucocorticoid-induced osteoporosis, which was characterized by decreased %Tb.Ar, Tb.Wi and Tb.N,while increased Tb.Sp exit in GIO rat model at the same time. As illustrated in Tab 3,in the groups treated with glucocorticoid plus RRP (4 or 7.5 $ml \cdot kg^{-1} \cdot d^{-1}$ oral), %Tb.Ar increased significantly compared with the GC group while Tb.Sp decreased significantly ($P<0.05$). The results above demonstrated that RRP can prevent glucocorticoid side effect on bone formation as well as the therapeutic measure of GIO include calcium, vitamin D. Moreover, RRP has been shown to stimulate the proliferation and differentiation of osteoblasts in vitro [12]. These results suggest that the bone protective effects of RRP in glucocorticoid-treated rats are likely due to the promotion of bone formation.

Bone is a composite material, both resilience and resistance exit in bone. Bone biomechaniacs parametes is the effective evaluation of bone quality. Modulus of rigidity represents the ability of bone resistant deformation, which is structural mechanics parameters in bone biomechaniacs parametes. The present study showed in GIO rats, the ability to resist the external force decreased and thus lead to increased fracture risk of bone. As shown in Tab 4, treated with glucocorticoid plus CaD or RRP (4 or 7.5 $ml \cdot kg^{-1} \cdot d^{-1}$ oral) can increase modulus of rigidity. Potential mechanism is

RRP improve bone quality by promoting bone formation, prevent osteoporosis in GIO rats, tthis is probably the mechanism that RRP inhibit the decreased modulus of rigidity induced by glucocorticoid.

In conclusion, results from the above study showed that RRP prevents glucocorticoid-induced osteoporosis by improving bone formation.

Acknowledgments

This study was supported by the Science and Technology Planning Project of Guangdong Province (No. 2011B031800229, 2011B060300032).

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