Journal of Animal and Veterinary Advances 13 (2): 66-71, 2014
ISSN: 1680-5593

Changes in Bone Mechanical Properties and Geometry are Associated with Age and Pseudopurpurin Deposition in Red-Boned Guishan Goats

[1, 2]Chen-Chen Wu, [1]Baoyu Zhao, [2]XiaoBing Li, [2]GuoWen Liu and [2]Zhe Wang
[1]College of Animal Science and Veterinary Medicine,
Northwest A&F University, 712100 Xi'an, China
[2]College of Animal Science and Veterinary Medicine,
Jilin University, 130062 Changchun, China

Abstract: Pseudopurpurin improves bone strength, structure and metabolic function in 18 months old red-boned goats in previous experiment. The objective of the study was to evaluate whether the greater pseudopurpurin deposition would result in better bone mechanical properties, geometry and architecture in red-boned goats with age. Bone mineral density, geometry, architecture and mechanical properties were analyzed using dual-energy X-ray absorptiometry, micro-computed tomography and materials-testing. The results showed that bone mineral densities, the micro-structural parameters and mechanical properties of the distal femur and femoral diaphysis in red-boned goats were not significantly different from common goats at 6 months of age. At 12 months of age, the micro-structural parameters and mechanical properties of the distal femur were better in red-boned than in common goats, the marrow area, cortical area and total area of the femoral diaphysis in red-boned goats were significantly different from common goats. The findings suggest that the pseudopurpurin was initially deposited in the cancellous bone and then in the cortical bone of the red-boned goats thus, the changes in bone parameters were seen earlier in the cancellous bone than the cortical bone. Both the pseudopurpurin deposit and the geometry and strength of the femur increased with age in red-boned goats.

Key words: Red-boned Guishan goats, pseudopurpurin, bone mineral density, bone mechanical properties, bone geometry

INTRODUCTION

Guishan goats are a rare wild breed of *Capra hircus* native to China and are currently categorized as first-class national protected animals in China. They are distributed mainly over the Guishan Mountains of Yunnan Province in the Southwest of China. These goats were developed in Yunnan Province over >20 generations of inbreeding and live mainly on grazing. Red-colored bones were first found in some Guishan goats in the 1980s and they were subsequently designated red-boned Guishan goats to distinguish them from common Guishan goats. Red-boned Guishan goats and common Guishan goats which do not have red bones are in fact the same species of Guishan goat (Gu *et al*., 2009, 2011; Tao *et al*., 1999) and both live and graze in the same location in the Guishan mountains. Through long-term observations, researchers found that the red color of the bones develops as the goats mature. In a previous study, at 18 months of age, all of the bones of red-boned Guishan goats are a carmine color. The whole cancellous bone is much more red in the red-boned Guishan goats than in the common Guishan goats. Researchers demonstrated that the red material in the bones of red-boned Guishan goats is pseudopurpurin which has a high affinity for the mineral salts of bone. At 18 months of age, the pseudopurpurin-containing bones of red-boned goats have increased bone mineral content and bone strength as well as structural and metabolic enhancements (Wu *et al*., 2012a, b). However, it remains unclear if the increased pseudopurpurin deposition and the improved mechanical, geometrical and architectural properties of the bones of red-boned goats persist with age. Therefore, to develop a better understanding of red-boned Guishan goats, their bone geometry, architecture and mechanical properties were compared with those of common Guishan goats using micro-computed tomography scanning and a materials-testing machine.

Corresponding Author: Baoyu Zhao, College of Animal Science and Veterinary Medicine, Northwest A&F University, 712100 Yangling, China

MATERIALS AND METHODS

Goats: Six common and six red-boned Guishan goats aged 6 and 12 months (n = 3 goats of each breed per age category) were randomly selected for use in this study. All goats were grazed under conventional conditions in the same environment with free access to feed and water.

The goats were euthanized with sodium pentobarbital and the left and right femur were excised showed the color of bone, cleaned of soft tissues, wrapped in saline-soaked gauze and stored at -20°C until analysis. The whole left femur was used for Dual Energy X-ray Absorptiometry (DEXA) studies. The left femoral diaphysis and the distal femur were used to submission for micro-CT analysis. The right femoral diaphysis and the distal femur were evaluated using a test of mechanical properties.

Dual energy X-ray absorptiometry: Prior to Dual Energy X Ray Absorptiometry scanning (DEXA, Medilink Osteocore3, France) the left femur was brought slowly to room temperature in a saline bath. Scanning was performed with each bone positioned on its caudal surface. BMD measeuements were performed in the trabecular bone of distal femur and cortical bone of femoral diaphysis using DEXA.

Bone micro-computed tomography scanning: The architectural characteristics of cancellous bone from the distal femoral metaphysis and cortical bone from the mid-femoral diaphysis were assessed using a desktop micro-CT System (eXplore Locus SP, GE Healthcare, USA). For image acquisition from samples from the distal femur the specimens were machined into 20 mm diameter cylinders. The specimens were scanned using a large tube 14 μm to 150 min-ss-microtomography scan protocol with a 14 μm isotropic voxel size. The scan protocol consisted of rotation through 210° at a rotation step of 0.4°, the X-ray settings were standardized to 80 kV and 80 μA and the exposure time was 2960 msec per frame. The samples were scanned continuously with a thickness and increment of 20 μm for 120 slices and scan time was approximately 150 min per sample. Three-dimensional morphometric analyses were performed in a 5×5×3 mm^3 ROI on the area of cancellous bone located 1-1.5 mm distal to the growth plate of the femur. Three-dimensional (3D) surface renderings were performed using Mic-view V 2.1.2 3D Reconstruction Software. The scan database was analyzed to give the 3D parameters for each ROI and the 3D ROIs were reconstructed and analyzed using the same thresholds. Three-dimensional histomorphometric analyses of samples from thawed femoral diaphyses were performed on 2 mm thick rings of mid-shaft cortical bone using the scanning protocol described above. The CT images were segmented into bone and marrow regions by applying the same, visually chosen, threshold for all samples.

The trabecular Bone Volume fraction (BV/TV) was calculated from the Bone Volume (BV) and total Tissue Volume (TV). Mean Trabecular Thickness (Tb.Th) was determined from the local thickness by the Distance-Transformation Method. Trabecular Separation (Tb.Sp) and Trabecular Number (Tb.N) were estimated on the basis of the plate model. The Structural Model Index (SMI) is a parameter used to quantify the characteristic form of a three-dimensionally described structure in terms of the plate-like or rod-like nature of the complete structure. The Connectivity Density (Conn.D) of the trabecular bone was also measured. Femoral cortical geometry was assesd using micro-CT at the mid-femoral diaphysis. Researchers measured the mean thickness, inner perimeter, outer perimeter, marrow area, cortical area and total area.

Assessment of mechanical properties: The specimens were thawed at room temperature and hydrated with saline prior to mechanical testing. For compression testing, 2×2×2 cm^3 cubes of cancellous bone from the right distal femur were cut with a motor wafer saw to obtain samples with parallel surfaces. These were then were placed centrally between two parallel steel plates attached to the Materials-Testing machine (MTS 858 System Inc., MN, USA). A compressive force was applied along the craniocaudal loading axis at a constant deformation rate of 2 mm min^{-1} until destruction of the sample. The right femoral diaphysis was subjected to a three-point bending test using the same materials-testing machine. For three-point bending, the femoral diaphysis was placed with its anterior surface facing upward on two lower support bars 40 mm apart and a force in the opposite direction was applied to the midpoint. The holders were perpendicular to the horizontal axis and the force was applied downward, perpendicular to the horizontal axis and at the midpoint of the specimen. A constant displacement rate of 2 mm min^{-1} was applied until destruction. The mechanical properties measured included maximum energy (U), the area of under the load-deformation curve, Elastic modulus (Eb), the linear region of the load-deformation curve, yield stress (σ_{yield}), the maximum point on the linear region of the curve and ultimate stress (σ_{ult}) (the maximum point on the curve). Ultimate strain (ε_{ult}) and yield strain (ε_{yield}) were measured when σ_{ult} and σ_{yield} were achieved.

Statistical analyses: All data are presented as mean values±Standard Deviation (SD). Significance was defined as $p<0.05$. All statistical analyses were performed using the statistical package SPSS for Windows Version 13.0. Differences between red-boned goats and common goats at 6, 12, 18 and 36 months of age were assessed using student's t-test.

RESULTS AND DISCUSSION

The color of bone in red-boned goat: At 6 months of age the bones are a normal color while by 12 months of age, the bones have developed a pale pink color. The surface of the marrow cavity or pink-stained in concentric rings around the marrow cavity which can be seen on the cross section of the bone at 12 months of age. However, all the cancellous bone of 12 months old red-boned goats are stained a carmine color compared to those of common goats (Fig. 1).

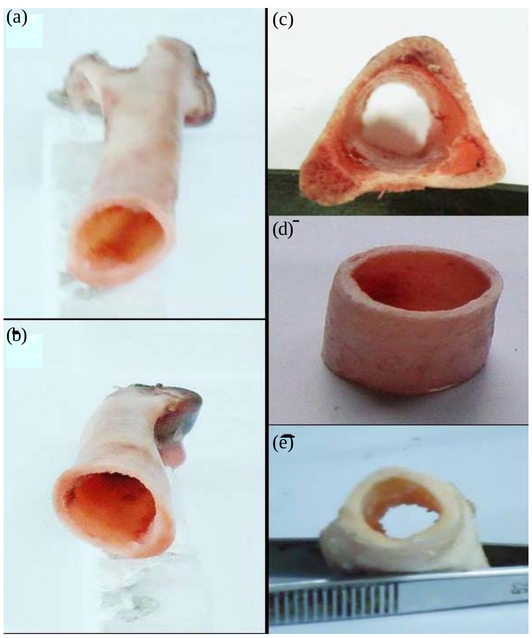

Fig. 1: a) Cross-section of the bone diaphysis from a red-boned goat at 6 months of age; b) cross-section of the femoral diaphysis from a common Guishan goat at 6 months of age; c) the surrounds the marrow cavity in the red-boned goat at 12 months of age; d) the cortical bone from a red-boned goat at 12 months of age; e) the cortical bone from a common goat at 12 months of age

Bone Mineral Density (BMD): The mean BMD, BMC and bone area of the femoral diaphysis and distal femur as determined by DEXA are shown in Table 1 and 2. There were no significant differences in BMD, BMC or bone area between red-boned goats and common Guishan goats at 6 and 12 months of age (p>0.05).

Bone mechanical properties: The mechanical properties of the femoral diaphysis of each goat were determined using a three-point bending test. In both red-boned and common Guishan goats, the maximum energy (U), Elastic modulus (Eb), yield stress (σ_{yield}), ultimate stress (σ_{ult}), yield strain (ε_{yield}) and ultimate strain (ε_{ult}) increased with age. At 6 and 12 months of age there were no differences between the bone mechanical properties of red-boned goats and common goats (p>0.05) (Table 3).

The distal femur of each goat was used in a compression test. In both red-boned and common Guishan goats, the maximum energy (U), Elastic modulus (Eb), yield stress (σ_{yield}), ultimate stress (σ_{ult}), yield strain (ε_{yield}) and ultimate strain (ε_{ult}) increased from 6-12 months of age. At 6 months of age, there were no significant differences in the mechanical properties of the distal femur of red-boned and common Guishan goats (p>0.05). At 12 months of age, the Eb and σ_{yield} were higher and ε_{yield} was lower in red-boned goats than in common goats (p<0.05). The σ_{ult} was higher and ε_{ult} was lower in red-boned goats than common goats however, the σ_{ult} and ε_{ult} were not significantly different (p>0.05) (Table 4).

Microstructural changes in the distal femoral metaphysis and mid-femoral diaphysis: The 3-D reconstruction of the bone structures using micro-CT images allowed quantification of trabecular and cortical bone changes in the femur. At 6 months of age, the trabecular Bone Volume fraction (BV/TV), mean

Table 1: Results of DEXA of the distal femurs of red-boned and common Guishan goats

Time period	Goats	Distal femur (BMD g cm^{-2})	Distal femur (BMC g)	Distal femur (Area cm^2)
6 months	Red-boned goats	0.201±0.01	2.18±0.095	10.84±2.11
	Common goats	0.182±0.02	1.83±0.098	10.06±2.21
12 months	Red-boned goats	0.305±0.03[a]	8.43±0.970[a]	23.74±3.32[a]
	Common goats	0.230±0.04[a]	4.98±0.960[a]	21.67±3.43[a]

*Significant difference between red-boned goats and common goats of the same age (p<0.05), determined by pairwise t-test; [a]Significantly different from the values for the goats at 6 and 12 of age, respectively (Bonferroni correction test)

Table 2: Results of DEXA of the femoral diaphyses of red-boned and common Guishan goats

Time period	Goats	Femur diaphysis (BMD g/cm^2)	Femur diaphysis (BMC g)	Femur diaphysis (Area cm^2)
6 months	Red-boned goats	0.521±0.030	16.33±0.87	31.34±2.13
	Common goats	0.519±0.040	16.89±0.92	32.54±2.45
12 months	Red-boned goats	0.903±0.090[a]	46.95±3.76[a]	51.99±7.71[a]
	Common goats	0.809±0.089[a]	42.92±3.12[a]	53.05±8.32[a]

*Significant difference between red-boned goats and common goats of the same age (p<0.05), determined by pairwise t-test; [a]Significantly different from the values for the goats at 6 and 12 of age, respectively (Bonferroni correction test)

Trabecular Thickness (Tb.Th), Trabecular Number (Tb.N), Trabecular Separation (Tb.Sp), Structural Model Index (SMI) and Connectivity Density (Conn.D) did not differ in the distal femurs of red-boned goats and common goats (p>0.05). At 12 months, BV/TV, Tb.Th, Tb.Sp and SMI in the distal femur were significantly greater in red-boned goats than in common goats (p<0.05). However, Tb.N and Conn.D did not differ (p>0.05) (Table 5).

At 6 months of age, the marrow area, cortical area, mean thickness, inner perimeter, outer perimeter and total area of the femoral diaphyses of red-boned goats did not differ from those of common goats (p>0.05). At 12 months of age, the marrow area, cortical area and total area of the femoral diaphyses of red-boned goats were all greater than in common goats (p<0.05). There were no differences between red-boned and common goats in the mean thickness, inner perimeter and outer perimeter of their femoral diaphyses at 12 months of age (p>0.05) (Table 6).

Table 3: Mechanical properties of the femoral diaphyses of red-boned and common goats as assessed by the three-point bending test

Mechanical properties	Red-boned goats 6 months	Red-boned goats 12 months	Common goats 6 months	Common goats 12 months
U(J)	6.11±0.61	8.21±0.81[a]	5.99±0.77	8.19±0.89[a]
Eb (Gpa)	1731.80±411.6	2738.80±311.9[a]	1699.60±432.5	2699.60±332.5[a]
σ_{yield} (MPa)	176.40±22.9	206.40±42.8[a]	171.30±21.9	203.80±43.1[a]
σ_{ult} (MPa)	231.50±28.5	291.50±31.5[a]	228.70±29.5	228.70±29.5[a]
ε_{yield} (%)	4.21±1.21	6.33±1.214[a]	4.16±1.22	6.16±1.77[a]
ε_{ult} (%)	6.66±0.87	8.62±0.84[a]	6.81±0.89	8.37±0.89[a]

Table 4: Mechanical properties obtained of the distal femurs of red-boned and common goats as assessed by compression testing

Mechanical properties	Red-boned goats 6 months	Red-boned goats 12 months	Common goats 6 months	Common goats 12 months
U (J)	1.99±0.47	2.89±0.54[a]	2.01±0.56	2.97±0.44[a]
Eb (Mpa)	371.40±40.2	472.22±41.3[a,*]	357.33±39.1	411.20±45.7[a]
σ_{yield} (MPa)	15.20±3.50	22.40±5.20[a,*]	15.10±3.40	17.30±5.10[a]
σ_{ult} (MPa)	19.30±5.80	26.10±8.20[a]	20.40±6.20	26.70±7.80[a]
ε_{yield} (%)	10.10±2.11	12.40±1.09[a,*]	11.50±2.44	16.40±1.11[a]
ε_{ult} (%)	14.70±1.31	18.43±1.55[a]	15.30±1.33	18.82±1.43[a]

*Significant difference between red-boned goats and common goats of the same age (p<0.05); determined by pairwise t-test; [a]Significantly different from the values for the same goats at 6 and 12 of age, respectively (Bonferroni correction test)

Researchers have previously reported that the red material in the bones of red-boned goats is pseudopurpurin. This is one of the main constituents of madder, a plant that is eaten by the goats. Pseudopurpurin has a high affinity for the mineral salts of bone and its presence results in a high level of mineral salts in the bone and consequent improvement in bone strength and enhancement of the structure and metabolic functions of the bone at 18 months of age (Wu *et al*., 2012b). In this study, researchers have observed the color of bone and demonstrated differences in the BMD, biomechanical properties and bone architecture of the femoral diaphyses and distal femurs of red-boned and common goats from 6-12 months of age.

BMD (areal BMD) as measured by DXA is a composite variable influenced by bone volume, bone size and bone matrix mineralization density (Roschger *et al*., 2008). BMD is influenced by a variety of factors including heredity, environment, nutrition, age, gender and skeletal site (Chen *et al*., 2008). BMD is an important parameter of bone quality, reflects the degree of bone mineralization and is an important predictor of fracture risk in bone humans. The BMC is expressed as ash mass per volume unit, ash or calcium fraction of dry weight, equivalent hydroxyapatite content per volume unit. Thus, determined, the amount of mineral is the mineral content

Table 5: The 3D microstructural properties of the trabecular bone of the distal femoral metaphyses of red-boned and common goats

Microstructural properties	Red-boned goats 6 months	Red-boned goats 12 months	Common goats 6 months	Common goats 12 months
BV/TV (%)	9.500±1.660	18.770±3.660[a,*]	7.650±1.560	9.440±3.010[a]
Tb.Th (mm)	0.187±0.040	0.221±0.050[a,*]	0.142±0.030	0.153±0.050[a]
Conn.D (/mm^3)	41.400±8.700	59.500±9.200[a]	38.500±8.800	53.300±9.500[a]
Tb.N (/mm)	2.650±0.340	3.210±0.230[a]	2.110±0.290	2.780±0.250[a]
Tb.Sp (mm)	0.100±0.011	0.140±0.013[a,*]	0.170±0.013	0.210±0.020[a]
SMI	0.207±0.015	0.211±0.014[a,*]	0.227±0.017	0.311±0.017[a]

Table 6: The 3D microstructural properties of the cortical bone of the mid-femoral diaphyses of red-boned and common goats

Microstructural properties	Red-boned goats 6 months	Red-boned goats 12 months	Common goats 6 months	Common goats 12 months
Mean thickness (mm)	2.330±0.48	2.9700±0.64[a]	2.270±0.41	2.5500±0.71[a]
Inner perimeter (mm)	19.50±3.78	26.700±4.11[a]	18.60±3.56	20.440±4.02[a]
Outer perimeter (mm)	40.20±4.66	50.500±6.78[a]	38.99±5.01	43.800±7.01[a]
Marrow area (mm^2)	58.50±7.11	88.760±11.3[a,*]	50.70±6.99	59.400±10.5[a]
Cortical area (mm^2)	98.32±11.4	126.33±16.8[a,*]	91.20±10.4	107.55±17.3[a]
Total area (mm^2)	170.9±21.5	199.40±28.4[a,*]	165.2±20.4	180.60±26.5[a]

*Significant difference between red-boned goats and common goats of the same age (p<0.05); determined by pairwise t-test; [a]Significantly different from the values for the same goats at 6 and 12 of age, respectively (Bonferroni correction test) of solid bone tissue (Akkus *et al*., 2003). The mean BMD and BMC of the femoral diaphyses and distal femurs of both red-boned and common goats did not differ at 6 and 12 months of age in this study. Both BMD and BMC were elevated in red-boned goats at 18 months of age (Wu *et al*., 2012b). These results indicate that bone mineral content and bone mineral density begin to improve in red-boned goats relative to common goats from the bone began to turn red.

BMD correlates to micro-architectural parameters and mechanical properties. The bone geometric construction and biomechanics affects BMD value (Katz and Meunier, 1993). Trabecular bone has a complex 3D structure that consists of interconnecting plates and rods of bone. An estimation of the plate-like or rod-like characteristic of the trabeculae is given by the SMI which strongly reflects the mechanical properties of the trabeculae. In this study, after 12 months of age the BV/TV, Tb.Th, Tb.Sp and SMI were significantly different in the distal femurs of red-boned goats compared with common goats. This is furthered by the fact that both SMI and BV/TV correlated very highly with Tb.Th and Tb.Sp and less so with Tb.N and Conn.D. Mechanistically, this indicates that an increase in bone volume results in an increase in trabecular diameter but does not cause an increase in trabecular number or connectivity (Mittra *et al*., 2005). A loss of bone causes the trabeculae to become rod-like while a net increase in bone volume causes them to become plate-like (Hildebrand and Ruegsegger, 1997; Brunader and Shelton, 2002). This interrelationship between trabecular mechanical and microstructural properties demonstrates the consequences of trabecular remodeling (Mittra *et al*., 2005). At 12 months of age, compression testing showed that the Eb, σ_{yield} and ε_{yield} in the distal femurs of red-boned goats were greater than in common goats. There are relationships between architecture and σ_{yield} and σ_{ult} stress as well. At 18 months of age, the Eb, σ_{yield}, σ_{ult}, ε_{yield} and ε_{ult} in distal femurs of red-boned goats were all better than in common goats (Wu *et al*., 2012b). Ultimate strength is better correlated with Tb.N than with Tb.Th, suggesting that the maintenance of trabeculae microarchitecture is more important for the strength of the bone than is a subsequent increase in the thickness of the remaining trabeculae (Genant *et al*., 1999). At the mid-femoral diaphysis, researchers assessed cortical bone geometry and strength by micro-CT and 3-point bending. Compared with common goats, the marrow area, cortical area and total area of the femoral diaphysis of red-boned goats were greater at all measurement times at 12 months of age. Micro-CT correlates with the mechanical properties of cortical bone because micro-CT is strongly related to ash density and ash density is correlated with the mechanical properties of cortical bone (Keller, 1994; Kaneko *et al*., 2003).

All the bone of 6 months old red-boned goats are normal color, around the marrow cavity and the cancellous bone of 12 months old red-boned Guishan goats are stained a carmine color compared to those of common goats. Therefore, some parameter of the bone microstructure and biomechanics in red-boned goats were significantly higher than common goats at 12 months of age. With age, adaptive changes in bone geometry leading to cortical expansion, endosteal resorption and periosteal bone apposition result in the equalization of mechanical properties because of increases in the areal and polar moments of inertia of the whole bone (Hahn *et al*., 1992; Bagi *et al*., 2006; Fukuda and Iida, 2004; Iida and Fukuda, 2002). The results demonstrate that bone microstructure and biomechanics improved first at the distal femur and then at the femoral diaphysis. This is similar to the pattern of pseudopurpurin deposition in the bone. In other words, the pseudopurpurin was deposited in the bone causing the bone to turn red. This increased the mineralization of the bone and caused corresponding changes in the bone's microstructure and mechanical properties.

Ossification presented radiation from central to around. Osteoclasts are then recruited and activated osteoclasts fuse to become multinucleated osteoclasts. Activated osteoclasts mediate the resorption of the underlying bone. Subsequently, osteoblasts are recruited to the resorption cavity and lay down new osteoid which eventually becomes calcified (Datta *et al*., 2008). Pseudopurpurin resembles alizarin because it forms a colored calcium salt that is deposited in bone (Richter, 1937). The data suggest that pseudopurpurin which is the red color in the bones of red-boned goats was first deposited in cancellous bone and then in cortical bone. The pseudopurpurin-calcium salt was initially formed in transitional trabecular bone near the growth plate. This would result in the improvement in the micro-structural and biomechanical properties that researchers observed first in the distal femur and then at the femoral diaphysis. Researchers suggest that it is the pseudopurpurin-calcium salt deposited in the bones of red-boned goats that improves their bone micro-structure, biomechanics and density.

CONCLUSION

In this study, researchers have demonstrated the pattern of improvements in bone quality parameters resulting from pseudopurpurin deposition. The results indicate that pseudopurpurin is first deposited in cancellous bone and then in cortical bone. This results in changes in bone quality becoming evident sooner in cancellous bone than in cortical bone. As pseudopurpurin deposits increase with age, the geometry and strength of the bone also increases in red-boned goats. These results provide insights into the effects of pseudopurpurin on bone mineralization in goats and may also help to further the understanding of the bone mineralization and bone quality in humans and other mammals.

ACKNOWLEDGEMENTS

This study was supported by the National Nature Science Foundation of China (31302153) and the origination fee of doctoral research (Z111021305). Z. Wang and G.W. Liu design the study, C.C. Wu, X.B. Li and J.G. Wang conduct of the study, C.C. Wu collect the data, C.C. Wu analysis the data, C.C. Wu and T.S. Han, drafting the manuscript, Z. Wang and G.W. Liu revised the manuscript content.

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