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ORIGINAL ARTICLE

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Effects on the bones of vanadyl acetylacetonate by oral administration: a comparison study in diabetic rats

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Abstract Oral delivery, rather than parenteral administration, would be beneficial for treating diabetic mellitus owing to the need for a long-term regimen. The objectives of this study were to evaluate oral delivery tolerance and the effects on the bone of accumulated vanadium following the long-term administration of vanadyl acetylacetonate (VAC). Normal and diabetic rats were intragastrically administered VAC at a dose of 3 mg vanadium/kg body weight once daily for 35 consecutive days. VAC did not cause any obvious signs of diarrhea, any changes in kidney or liver, or deaths in any group. The phosphate levels in the bone were slightly increased, and the calcium levels in the bone were not obviously changed as compared with those of the rat group not receiving VAC. After administration of VAC, the decreased ultimate strength, trabecular thickness, mineral apposition rate, and plasma osteocalcin in diabetic rats were either improved or normalized, but reduced bone mineral density (BMD) in diabetic rats was not improved. None of the parameters evaluated in normal rats were altered. The results indicate that the oral VAC is tolerated and benefits the diabetic osteopathy of rats, but seems not to influence the bone of normal rats. They also suggest that VAC improves diabetes-related bone disorders, primarily by improving the diabetic state.

Key words vanadyl acetylacetonate · diabetes · bone markers · bone biomechanics · bone histomorphometry

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Introduction

Vanadyl acetylacetonate (VAC) is an organic type of vanadium compound which has exhibited insulin-mimetic effects in type 1 and type 2 diabetic animals [1–3] and human subjects [4–6], and prevented some diabetes-induced complications in animals [7,8]. Other pharmacological activities of VAC include the inhibition of gluconeogenesis [9], a decrease in glutamate dehydrogenase activity [10], and antilipolysis [11].

Oral delivery, rather than parenteral administration, would be beneficial for treating diabetic mellitus owing to the need for a long-term regimen. Accordingly, we need to know what happens when VAC is administered orally, in particular for long-term administration. Vanadium mainly exists in +4 and +5 compounds in the biological body. Vanadate (+5) and vanadyl (+4) have shown poor absorption from the gastrointestinal (GI) tract and GI side-effects. Therefore, various organic vanadium compounds, i.e., bis(maltolato)oxovanadium (BMOV), vanadyl sulfate (VS), vanadyl 3-ethylacetylacetonate (VET), and VAC, have been synthesized or re-evaluated in order to improve absorption and safety [12]. VAC with an organic ligand is more effective for lowering plasma glucose levels in diabetic animals compared with other vanadium compounds, including BMOV, VS, and VET [13]. In these studies, however, these vanadium complexes were given with drinking water, and the actual dose and duration of administration remained unclear.

Furthermore, the long-term administration of a vanadium compound to rats resulted in a significant vanadium accumulation in the bone. The accumulated concentration of vanadium in the bone was reported to be approximately 6–10 times higher than that in the liver [14,15], and the elimination half-life of vanadium was up to 723 h (30 days) [16–18], indicating that this element eliminates from bone very slowly. Therefore, this accumulation may be potentially toxic to the bone.

Our previous pharmacokinetic studies [19] showed that VAC was widely distributed in various tissues and accumu-

lated most in rat femur tissue. The average elimination half-life values of vanadium in femur tissue were up to 657.3 ± 77.9 h (27.4 ± 3.2 days), which were similar to those of the other vanadium compounds mentioned above. Nevertheless, unlike other vanadium compounds, VAC seemed to be better tolerated, and no obvious toxic signs, such as diarrhea, were observed over several days. However, the tolerance for longer-term oral administration of VAC and its effects on the accumulation on the bone are still unknown. These may be the important issues which need to be solved before any potential clinical therapy for diabetes can be considered.

Diabetic osteopathy, as one of the diabetes-induced complications, leads to diminished bone formation [20], retardation of bone healing [21], and osteoporosis [22]. Bone mineral density (BMD) [23] and biomechanical integrity [24] are referential predictors of fracture, and patients with type 1 diabetes incur a higher incidence of fractures than healthy individuals. Therefore, BMD and histomorphometry were used to evaluate the potential of the drug to prevent the osteopenia associated with type 1 diabetes [25,26]. The structural integrity of the femur or tibia was also examined in order to evaluate the biomechanical consequences of diabetes [24]. The results from an in vitro model using osteoblast-like cells showed that vanadium exerted biphasic effects: a low concentration of vanadium stimulated osteoblast proliferation and differentiation, but a high concentration inhibited these effects [27–29]. Nevertheless, in vivo evaluation data on bone in the presence of vanadium are still unavailable.

The objectives of this study were to observe the tolerance, such as loss of body weight or diarrhea, and the effects of VAC on bone formation/resorption parameters, density, biomechanics, and histomorphology following oral administration of VAC once daily for 35 consecutive days in normal and diabetic rats.

Materials and methods

Animals and reagents

Ten-week-old male Sprague–Dawley rats, weighing 210–240 g, were obtained from the Experimental Animal Center of Peking University and maintained in a light/dark cycle. All animals were allowed free access to standard rat chow and water. The temperature and relative humidity were maintained at 20°C and 50%, respectively. The rats were acclimatized for 7 days prior to the induction of diabetes. All the animal experiments adhered to the principles of care and use of laboratory animals, and were approved by the Institutional Animal Care and Use Committee of Peking University.

VAC was prepared and purified according to a previous report [30]. Alloxan was purchased from (Sigma-Aldrich, St. Louis, MO, USA). All other chemicals used were of analytical grade and were commercially available.

Experimental design

Induction of diabetes

Normal Sprague–Dawley rats were fasted for 12 h before the induction of diabetes. Type 1-like diabetes was induced by the intraperitoneal (i.p.) injection of freshly prepared alloxan (150 mg/kg) in physiological saline for 2 consecutive days (once daily). Blood samples for an analysis of plasma glucose were collected 48 h after injection of the alloxan solution, and plasma glucose levels were assayed using plasma glucose kits (Zhongsheng Biotech, Beijing, China). Rats with a plasma glucose level ≥13 mmol/l were used as diabetic rats, and diabetic rats with hyperglycemia (≥13 mmol/l) for 7 consecutive days were included in the experiments.

Administration

Twenty normal rats were divided equally into two groups: *Group Norm* and *Group Norm-VAC*. The rats in *Group Norm* were given physiological saline as the normal control, and the rats in *Group Norm-VAC* were given VAC at a dose of 3 mg vanadium (V)/kg as a treated normal control. Similarly, 20 diabetic rats were divided equally into two groups: *Group Diab* and *Group Diab-VAC*. The diabetic rats in *Group Diab* were given physiological saline as the diabetic control, and the diabetic rats in *Group Diab-VAC* were given VAC at a dose of 3 mg V/kg. From day 10 after the alloxan injection, VAC or physiological saline was given to rats intragastrically once daily for 35 consecutive days. Tetracycline (Sigma) dissolved in physiological saline was administered i.p. to all rats at a dose of 20 mg/kg 14, 13, 4, and 3 days before they were killed on day 35. The mean age of the rats at the end of the study was 17.5 weeks. During the experimental period, body weight and diarrhea were monitored.

Sampling and measurement

In all groups, blood samples were collected via the tail vein on the day before dosing, and on days 7, 14, 21, 28, and 35 after the dose administration, and then immediately put into heparinized capillary tubes. The blood was centrifuged at 3000×*g* for 10 min, and the plasma was collected. Plasma glucose levels were measured weekly using the glucose–oxidase method. Plasma samples on days 0, 14, and 35 were measured for an evaluation of indicators including calcium (Ca), phosphate (P), tartrate-resistant acid phosphatase (TRACP), alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CRE), cholesterol (CHOL), triglyceride (TRIG), and osteocalcin (OC). The accuracy and precision of the evaluation of Ca, P, ALT, AST, CRE, CHOL, and TRIG met the specifications of biochemical kits purchased from Zhongsheng Biotech. Plasma TRACP was determined by a colorimetric method with *p*-nitrophenyl phosphate as substrate [31], and the relative standard deviations (RSDs) of the intra-assay and inter-assay were 2.7% and 8.1%, respec-

tively. Plasma ALP was assayed by colorimetry with *p*-nitrophenyl phosphate as substrate [32], and the RSDs of the intra-assay and inter-assay were 1.9% and 3.5%, respectively. Plasma OC was measured using a radioimmunoassay kit with rat OC as standard [33] obtained from the China Atomic Energy Research Institute (Beijing, China), and the RSDs of the intra-assay and inter-assay were 7.2% and 8.9%, respectively.

Blood samples on day 35 were used for the analysis of hematological indices, including erythrocyte count, leukocyte count, platelet count, hemoglobin, and hematocrit. On day 35 after treatment, all animals were killed and some tissues, including kidney, liver, pancreas, adipose tissue, tibia, and femur, were harvested. The calcium and phosphate levels in the femurs were measured using biochemical kits supplied by Zhongsheng Biotech on a Hitachi 7170A automatic analyzer (Instrument Hitachi, Tokyo, Japan).

Histopathological observations

On day 35, the kidneys, liver, pancreas, adipose tissue, and tibia were immediately collected from all the dead rats and fixed with 10% buffered neutral formalin, with the exception of the pancreata. They were then placed in Bouin's solution, embedded in paraffin, and cut into 5-μm slices. The slices were stained with hematoxylin and eosin for histological observations. In addition, the tibias were decalcified with 5% nitric acid before being embedded in paraffin.

Dual-energy X-ray absorptiometry

The excised femurs were placed on polystyrene trays to mimic soft tissues, and were examined by dual-energy X-ray absorptiometry using a Norland bone densitometer (XR-36 Mark II, Norland, Fort Atkinson, WI, USA). Measurements of BMD and bone mineral content (BMC) were performed using specialized software for small animals, and identical scan parameters (pixel size 1.0 × 1.0 mm, scan speed 60 mm/s) were used for all measurements. BMD and BMC were automatically calculated and expressed as mg/cm^2 and mg, respectively. The relative standard deviation for BMD and BMC measurements was 0.58% and 0.79%, respectively.

Biomechanical testing

The biomechanical properties of the fresh femurs were determined with a three-point bending test using a bone strength measuring apparatus (Model WD-1, Changchun Testing Machine, Changchun, China), as previously described [34]. Briefly, the bones were uniformly positioned on the loading stage. The distance between the two points on the loading apparatus was 25 mm. A breaking force was applied perpendicularly to the femoral diaphysis at a constant deformation rate of 2 mm/min. A load–deformation curve was recorded for each specimen via a computerized monitor linked to the tester. The ultimate strength (maximal load before the bone breaks, expressed in N), stiffness (maximal slope of the linear part of the curve, expressed in N/mm), and energy absorbed by the bone tissue (area under the load–deformation curve before the bone breaks, expressed in mJ) were obtained automatically.

The necks of the femurs were fractured in the same apparatus as described above. The distal ends of the femurs were fixed by clamps, and the femoral necks were tested with a vertical load applied to the top of the femoral heads. The test speed was 2 mm/min. Identical parameters (namely, ultimate strength, stiffness, and energy absorption) were recorded.

Bone histomorphometry

The right proximal tibial metaphysis was dissected away from the soft tissue, and fixed in 10% buffered neutral formalin for 48 h at 4°C. The bones were washed using water, dehydrated using gradual ethyl alcohol, embedded in a methylmethacrylate-based medium at room temperature for 6 days, and subsequently dried at 35°C until completely stiff. Sections 5-μm thick were obtained in the frontal plane using a microtome (Reichert-Jung Polycut S, Cambridge Instruments, NuBloch, Germany) and were stained with toluidine blue. Sections 10-μm thick were cut and mounted (but not stained) for tetracyclin fluorescence evaluation. Five sections per bone were evaluated for histomorphometry with a Q550-CW image analyzer (Leica, Heidelberg, Germany), as previously reported [34,35]. Standard terms were used according to standardized nomenclature [36]. Bone parameters included trabecular thickness (Tb.Th) (μm), osteoid thickness (O.Th) (μm), trabecular separation (Tb.Sp) (μm), and mineral apposition rate (MAR) (μm/day). The MAR was calculated by dividing the distance between two fluorescent bands (observed under ultraviolet light) by the number of days between the two labels.

Statistical analysis

Data were expressed as mean±standard deviation (SD). The analysis of variance (ANOVA) was used to determine significance among groups, after which post-hoc tests with the Bonferroni correction were used for comparisons between individual groups. A value of $P < 0.05$ was considered to be significant.

Results

Plasma glucose, body weight, and diarrhea

The average plasma glucose concentration in diabetic rats not treated with VAC maintained a higher level, ranging from 19.3 ± 3.8 to 23.7 ± 2.3 mmol/l. However, the average plasma glucose levels in diabetic rats treated with VAC declined from 21.8 ± 3.1 on the initial day to 7.2 ± 2.5 mmol/l on day 7. After that, the plasma glucose values varied in the normal physiological range from 6.0 ± 1.2 to 7.3 ± 1.1 mmol/l. The average plasma glucose concentration in normal rats

Table 1. Body weight and plasma levels of calcium (Ca), phosphate (P), alkaline phosphatase (ALP), osteocalcin (OC), tartrate-resistant acid phosphatase (TRACP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CREA), cholesterol (CHOL), and triglyceride (TRIG) in rats on day 0 (prior to dosing), and days 14 and 35 following intragastric administration of physiological saline or vanadyl acetylacetonate (VAC) once daily for 35 consecutive days. Data are expressed as mean ± standard deviation (n = 10)

Parameter	Day 0				Day 14				Day 35			
	Group Norm	Group Norm-VAC	Group Diab	Group Diab-VAC	Group Norm	Group Norm-VAC	Group Diab	Group Diab-VAC	Group Norm	Group Norm-VAC	Group Diab	Group Diab-VAC
Body weight (g)	246.7 ± 6.9	246.3 ± 7.4	218.0 ± 15.2	217.4 ± 14.9	297.7 ± 13.0	284.0 ± 15.4	192.3 ± 9.4	204.9 ± 10.3	304.5 ± 9.1	293.1 ± 12.7	215.3 ± 9.3	235.8 ± 11.4*
Ca (mg/dl)	11.43 ± 0.31	11.53 ± 0.29	11.59 ± 0.46	11.28 ± 0.88	12.10 ± 0.36	11.68 ± 0.19	11.78 ± 0.76	11.67 ± 0.53	11.00 ± 0.20	11.40 ± 0.34	11.63 ± 1.17	11.52 ± 0.79
P (mg/dl)	9.04 ± 0.66	9.17 ± 0.55	9.24 ± 1.28	9.38 ± 1.22	8.90 ± 0.53	9.03 ± 0.48	9.15 ± 1.23	9.08 ± 1.63	8.98 ± 0.44	8.86 ± 1.78	9.12 ± 1.38	8.85 ± 1.57
ALP (U/l)	333.2 ± 39.2	347.3 ± 46.1	449.8 ± 30.7	452.7 ± 55.4	344.4 ± 27.3	355.9 ± 42.2	411.4 ± 37.9	408.1 ± 24.0	339.3 ± 29.8	356.2 ± 28.4	441.4 ± 36.8	423.1 ± 33.6
OC (ng/ml)	15.62 ± 1.50	15.25 ± 1.32	12.03 ± 1.52	12.25 ± 1.14	15.22 ± 2.21	15.58 ± 3.30	9.28 ± 1.54	12.22 ± 1.36*	15.48 ± 4.45	15.11 ± 1.83	7.56 ± 1.92	12.61 ± 1.08*
TRACP (U/l)	2.51 ± 0.33	2.65 ± 0.30	2.55 ± 0.56	2.49 ± 0.27	2.44 ± 0.49	2.51 ± 0.24	3.15 ± 0.52	3.04 ± 0.17	2.65 ± 0.37	2.73 ± 0.55	3.46 ± 0.22	3.29 ± 0.38
ALT (U/l)	43.3 ± 5.1	40.4 ± 7.2	52.5 ± 10.1	58.7 ± 11.8	47.0 ± 9.9	44.3 ± 9.6	64.0 ± 8.2	52.8 ± 9.4*	43.2 ± 11.6	42.2 ± 10.3	59.8 ± 9.8	48.9 ± 10.2*
AST (U/l)	93.7 ± 10.7	90.9 ± 11.5	103.5 ± 14.0	107.2 ± 16.9	87.7 ± 7.4	92.9 ± 7.6	107.9 ± 15.3	100.9 ± 13.2	76.3 ± 12.7	74.4 ± 6.1	105.6 ± 16.7	95.6 ± 15.6
CRE (mg/dl)	0.33 ± 0.04	0.33 ± 0.02	0.59 ± 0.15	0.54 ± 0.16	0.29 ± 0.03	0.28 ± 0.07	0.53 ± 0.18	0.45 ± 0.09	0.29 ± 0.05	0.34 ± 0.09	0.60 ± 0.08	0.42 ± 0.14*
CHOL (mg/dl)	64.8 ± 14.4	68.4 ± 12.5	87.4 ± 9.5	87.4 ± 8.6	64.0 ± 12.1	66.6 ± 12.8	83.1 ± 12.6	61.2 ± 17.3*	64.5 ± 14.3	64.8 ± 13.4	86.0 ± 11.0	61.8 ± 7.8*
TRIG (mg/dl)	66.7 ± 10.9	64.4 ± 12.9	152.8 ± 15.9	154.6 ± 13.9	62.0 ± 12.6	60.7 ± 10.6	156.1 ± 15.9	61.7 ± 16.8*	69.3 ± 5.4	66.3 ± 11.2	162.8 ± 22.7	58.1 ± 16.2*

Notes: Normal rats in *Group Norm* were given physiological saline and normal rats in *Group Norm-VAC* were given VAC at a dose of 3 mg V/kg. Diabetic rats in *Group Diab* were given physiological saline and diabetic rats in *Group Diab-VAC* were given VAC at a dose of 3 mg V/kg. VAC or physiological saline was given to rats intragastrically once daily for 35 consecutive days
* $P < 0.05$ vs. *Group Diab* at the same sampling time point

Fig. 1. Mean plasma glucose level–time profiles after intragastric administration of physiological saline or vanadyl acetylacetonate (VAC) at a dose of 3 mg V/kg once daily for 35 consecutive days. Data are expressed as mean±standard deviation (n = 10). *Circles*, Group Norm; *squares*, Group Norm-VAC; *triangles*, Group Diab; *X-marks*, Group Diab-VAC. *Asterisk*, $P < 0.05$ Group Diab-VAC vs. Group Diab

receiving VAC was from 5.42 ± 1.19 to 7.44 ± 0.64 mmol/l, and was not significantly different from that in rats receiving saline, which ranged from 5.40 ± 0.41 to 7.24 ± 1.45 mmol/l, showing that VAC did not affect the plasma glucose levels in normal rats, as shown in Fig. 1.

Body weight was not significantly different between normal rats receiving VAC and normal rats not receiving VAC (*Group Norm-VAC* vs. *Group Norm*), but body weight in both normal rat groups (*Group Norm* and *Group Norm-VAC*) was considerably higher ($P < 0.05$) than that in diabetic rat groups (*Group Diab* and *Group Diab-VAC*) at the same time point. On day 35 after dosing, body weight in diabetic rats treated with VAC was markedly higher ($P < 0.05$) than that in diabetic rats not treated with VAC (*Group Diab-VAC* vs. *Group Diab*), as shown in Table 1. During the period of treatment, no obvious signs of diarrhea and no deaths were found in any group.

Plasma biochemical indicators

The plasma biochemical indicators are summarized in Table 1. On days 14 and 35, no evident differences in the levels of Ca, P, ALP, and TRACP were found between rats treated with VAC and the corresponding controls treated with physiological saline (*Group Norm-VAC* vs. *Group Norm*, *Group Diab-VAC* vs. *Group Diab*). The levels of ALP and TRACP in diabetic rats (*Group Diab* and *Diab-VAC*) were significantly higher ($P < 0.05$) than those in normal rats (*Group Norm* and *Norm-VAC*) at the same time point. Plasma OC concentrations in diabetic rats not receiving VAC (*Group Diab*) were markedly decreased ($P < 0.05$) compared with those in normal rats not receiving VAC (*Group Norm*). After diabetic rats were treated with VAC on days 14 and 35, the plasma OC levels of the diabetic rats (*Group Diab-VAC*) increased, and tended toward the values observed in normal rats.

The levels of CHOL and TRIG in diabetic rats treated with VAC (*Group Diab-VAC*) were markedly reduced ($P < 0.05$) compared with those in diabetic rats not treated with VAC (*Group Diab*), and eventually reverted to the range observed in normal rats (*Group Norm*). The plasma ALT and CRE levels in diabetic rats receiving VAC (*Group Diab-VAC*) were clearly lower ($P < 0.05$) than those in diabetic control rats (*Group Diab*), and were close to those in normal rats (*Group Norm* and *Norm-VAC*). The plasma AST levels in diabetic rats either treated with VAC or untreated were not significantly different (*Group Diab-VAC* vs. *Diab*). Furthermore, VAC treatment did not obviously change the plasma ALT, AST, and CRE levels in normal rats (*Group Norm-VAC* vs. *Group Norm*).

Blood indices

Hematological analysis showed a significant decrease ($P < 0.05$) in the erythrocyte count in rats receiving VAC (*Group Norm-VAC* and *Group Diab-VAC*) compared with corresponding control rats (*Group Norm* and *Group Diab*), and this was accompanied by an obvious increase in the leukocyte count. Furthermore, the leukocyte counts in diabetic controls (*Group Diab*) were distinctly lower ($P < 0.01$) than those in normal controls (*Group Norm*). No pronounced differences were observed in the platelet count, hemoglobin, and hematocrit between *Groups Norm* and *Norm-VAC* or between *Group Diab* and *Diab-VAC*, as shown in Table 2.

Histopathological observations

Histopathological analysis showed that no obvious differences could be observed in the kidneys, livers, pancreata, adipose tissue, and tibias from normal rats either untreated (*Group Norm*) or treated with VAC (*Group Norm-VAC*). However, in the kidneys, pancreata, and tibias from untreated diabetic rats, some morphological abnormalities were found, which mainly included atrophy on the surface of the renal capsules, multifocal glomerulus, renal tubule analosis, atrophy of pancreatic islands, a significantly reduced number of bone trabecula, and narrowing trabecula. In all the micrographs of the pathological slices taken, no obvious morphological abnormalities were observed in the livers or the adipose tissue.

Calcium and phosphate in bone

The femoral P levels in normal and diabetic rats treated with VAC were slightly increased compared with those in rats not treated with VAC (*Group Norm-VAC* vs. *Group Norm*, *Group Diab-VAC* vs. *Group Diab*), and the femoral Ca levels in normal and diabetic rats treated with VAC were not obviously changed compared with those in rats not treated with VAC (*Group Norm-VAC* vs. *Group Norm*, *Group Diab-VAC* vs. *Group Diab*), as indicated in Table 2. The Ca/P ratios were 2.17 ± 0.47 for *Group Norm*, 1.99 ± 0.29 for *Group Norm-VAC*, 2.07 ± 0.32 for *Group Diab*, and 1.89 ± 0.35 for *Group Diab-VAC*.

Table 2. Hematological indices in total blood, calcium (Ca), and phosphate (P) levels in femurs, bone mineral density (BMD), and biomechanical and histomorphometric values in the rats on day 35 following intragastric administration of physiological saline or vanadyl acetylacetonate (VAC) once daily for 35 consecutive days. Data are expressed as mean ± standard deviation ($n = 10$)

	Group Norm	Group Norm-VAC	Group Diab	Group Diab-VAC
Hematological indices				
Erythrocyte count ($\times 10^{12}$/l)	7.37 ± 0.33	6.62 ± 0.22*	7.66 ± 0.27	7.04 ± 0.22**
Leukocyte count ($\times 10^{9}$/l)	8.67 ± 0.16	9.52 ± 0.27*	7.34 ± 0.27	8.37 ± 0.28**
Platelet count ($\times 10^{12}$/l)	1.402 ± 0.162	1.438 ± 0.159	1.136 ± 0.161	1.186 ± 0.110
Hemoglobin (g/l)	162.5 ± 6.6	158.8 ± 11.1	163.0 ± 7.5	156.5 ± 13.1
Hematocrit	0.452 ± 0.019	0.453 ± 0.027	0.461 ± 0.034	0.459 ± 0.028
Ca (mg/g)	55.54 ± 15.53	54.95 ± 12.18	49.75 ± 14.16	49.82 ± 14.64
P (mg/g)	26.01 ± 3.79	28.14 ± 3.51	23.56 ± 4.39	26.64 ± 4.51
BMD (mg/cm^2)	129.4 ± 6.9	126.6 ± 3.7	117.6 ± 5.6	120.4 ± 4.8
BMC (mg)	128.2 ± 17.0	125.6 ± 13.9	113.9 ± 17.7	119.1 ± 19.1
Femoral diaphysis				
Ultimate strength (N)	76.7 ± 9.3	76.0 ± 9.6	56.5 ± 7.8	67.1 ± 10.0**
Stiffness (N/mm)	130.1 ± 18.1	126.6 ± 28.3	115.9 ± 27.5	114.7 ± 23.9
Energy absorption (mJ)	36.0 ± 8.1	34.4 ± 7.3	30.3 ± 6.2	30.6 ± 6.6
Femoral neck				
Ultimate strength (N)	88.1 ± 18.1	88.1 ± 8.2	60.5 ± 9.3	69.7 ± 7.4**
Stiffness (N/mm)	97.2 ± 22.1	95.5 ± 18.0	82.1 ± 15.7	86.7 ± 11.5
Energy absorption (mJ)	82.5 ± 26.7	81.9 ± 28.5	69.3 ± 17.2	70.2 ± 20.2
Histomorphometry				
Tb. Th (μm)	5.834 ± 0.827	6.082 ± 1.214	4.919 ± 0.543	5.637 ± 0.636**
O. Th (μm)	0.446 ± 0.050	0.414 ± 0.045	0.479 ± 0.049	0.444 ± 0.028
Tb. Sp (μm)	12.897 ± 1.583	12.943 ± 1.827	14.734 ± 2.117	14.911 ± 2.362
MAR (μm/day)	0.697 ± 0.181	0.684 ± 0.133	0.450 ± 0.158	0.647 ± 0.081**

* $P < 0.05$ vs. *Group Norm*; ** $P < 0.05$ vs. *Group Diab*

Dual-energy X-ray absorptiometry

No significant differences were observed in BMD values between normal rats receiving VAC and normal rats not receiving VAC (*Group Norm-VAC* vs. *Group Norm*). The BMD values in diabetic rats not treated with VAC were significantly decreased ($P < 0.05$) compared with those in normal rats not treated with VAC (*Group Diab* vs. *Group Norm*). Reduced BMD in diabetic rats was not improved by VAC treatment (*Group Diab-VAC* vs. *Group Diab*), as shown in Table 2. The results for BMC were similar to those for BMD (Table 2).

Biomechanical testing

There were no significant differences in any of the biomechanical parameters in normal rats either receiving or not receiving VAC. The ultimate strengths in the femoral diaphyses and the femoral necks in diabetic rats not treated with VAC were significantly decreased (approximately 26% for femoral diaphysis and 31% for femoral neck, on average 29%, $P < 0.05$) compared with those in normal rats not treated with VAC (*Group Diab* vs. *Group Norm*). After the 35-day treatment with VAC, the ultimate strengths in diabetic rats receiving VAC were significantly increased ($P < 0.05$) compared with those in diabetic rats not receiving VAC (*Group Diab-VAC* vs. *Group Diab*). However, no differences were observed in the stiffness and the energy absorption in diabetic rats either treated or not treated with VAC. In addition, the stiffness and the energy absorption in diabetic rats either receiving or not receiving VAC were slightly decreased compared with those in normal rats not receiving VAC (*Group Diab-VAC* vs. *Group Norm*, *Group Diab* vs. *Group Norm*), as shown in Table 2.

Bone histomorphometry

No significant differences in any bone histomorphometric parameters were observed in normal rats either receiving or not receiving VAC. Tb.Th and MAR values in the diabetic rats not treated with VAC were significantly lower ($P < 0.05$) than those in normal control rats (*Group Diab* vs. *Group Norm*), and both parameters in diabetic rats treated with VAC were markedly increased (*Group Diab-VAC* vs. *Group Diab*), while the O.Th and Tb.Sp values in all groups, namely, *Group Norm*, *Group Norm-VAC*, *Group Diab*, and *Group Diab-VAC*, were not significantly different, as shown in Table 2.

Discussion

The effectiveness of VAC for lowering plasma glucose levels in diabetic animals was proven [13], and that of vanadyl sulfate (VS) and sodium metavanadate (SMV) was demonstrated in diabetic animals and a limited number of patients [2,5,6]. Further investigation into an organic form of vanadium VAC is needed because VAC has been shown to have improved oral absorption and fewer side effects, such as diarrhea, than inorganic vanadium (e.g., VS, SMV). Our previous investigations showed that VAC could be absorbed well following oral administration [19]. As mentioned in the introduction section, the tolerance for longer-term oral administration of VAC and the effects of its accumulation on the bone may be the important issues for any potential clinical therapy for diabetes.

The vanadium may be absorbed into the bone or involved in the biomineralization process of the bone like Ca and P elements. It has been postulated that bone was an active vanadium accumulator, and the high skeletal retention was considered to be due to its rapid exchange with bone phosphate [37]. In the present study, however, P levels in the femurs of normal and diabetic rats treated with VAC were not significantly lower than those of the corresponding control rats not treated with VAC. Furthermore, plasma P levels in treated rats were not obviously increased when compared with those in untreated rats. These results suggest that the accumulated vanadium in bone may be incorporated into the bone instead of the "exchange," as described above. Otherwise, the P levels in the plasma would increase and those in the bone should drop. In fact, a slight increase in P content and a decrease in the Ca/P ratio were observed in the bones of rats treated with VAC, suggesting that vanadium accumulated in bone did not displace P, but may directly participate in the bone biomineralization process. The slight increase in P content may be explained by the assumption that the binding affinity of PO_4^{3-} to V^{4+} is higher than that to Ca^{2+}, thus leading to a slight increase in P in the bone. The vanadium may attract more P from the blood or other tissues. In fact, the slight reduction in P levels in plasma seemed to support this theory.

Type 1 diabetes is associated with a modest reduction in the BMD, bone strength, and bone formation [8,23,38]. The BMD at the site of the femurs in the diabetic rats treated with VAC was not significantly different from that in the untreated diabetic rats, but was markedly lower than that in the normal rats either treated or untreated, suggesting that vanadium did not affect the BMD values. Facchini et al. [39] also reported that bis(ethylmaltolato)oxovanadium (BEOV) did not alter the BMD between normal rats with or without BEOV and diabetic rats with or without BEOV, and the BMD values in diabetic rats after treatment with BEOV were still significantly lower than those in normal rats. The reduced BMD values in diabetic rats were simply the result of the diabetes. Therefore, vanadium did not seem to improve the loss of bone mass significantly, as BMD was one of the indicators for bone mass.

Many studies have shown that the BMD alone may not be sufficient to evaluate the strength of bone, and that the biomechanical properties of bone and trabecular structure may also be important factors that determine bone strength and individual risk of fractures [40–42]. Therefore, we further evaluated the bone strength of the femur by biomechanical testing, and that of the trabecular structure by histomorphometry.

The significant reductions in the ultimate strength of diabetic bones were derived from the diabetes, and the present results are similar to those in previous publications reporting that the ultimate bone strength of diabetic rats was reduced by 20%–67% compared with that of normal controls [38,43]. The ultimate bone strength in diabetic rats treated with VAC was significantly increased compared with that in untreated diabetic rats, indicating that VAC treatment may benefit the ultimate strength of bone in diabetic rats, as shown in insulin therapy [25,44]. With regard to the effects of VAC on the stiffness and energy absorption of bone, the results of the present study indicate that VAC seemed to have no obvious affect on the stiffness or the energy absorption when comparing diabetic rats receiving VAC to diabetic control rats, or comparing normal rats receiving VAC to normal control rats. Furthermore, the stiffness and energy absorption values between diabetic and normal control rats were not obviously different, and the results were very similar to those of a previous report [45] that no apparent changes in the stiffness of femurs were observed in spontaneously diabetic rats. However, it was reported that both stiffness and energy absorption values [24] in diabetic rats were significantly decreased compared with these values in normal rats. The differences between our results and the results reported previously may be due to variations in methodologies or the strain/breed of rats used in each study.

Extensive evidence has demonstrated that untreated or poorly treated diabetes is associated with low bone formation in both humans and experimental animals. In our findings, these results were confirmed, and the lowered values of Tb.Th and MAR in diabetic rats were converted to normal ranges after the rats were treated with VAC, indicating that VAC therapy would be beneficial for the correction of abnormal bone formation in diabetic rats. However, the O.Th and Tb.Sp values in diabetic and normal rats in our observations were not significantly different. It was previously reported [46] that Tb.Sp values appeared to be higher in diabetic rats than in normal rats. This difference may be due to the longer interval between diabetes induction and bone analysis in the reported studies (14 weeks in the reported study vs. 5 weeks in our design). The O.Th values in diabetic rats are not available in the literature.

OC and ALP values were the indicators of osteoblast activity, and the TRACP value was a marker of osteoclast activity. Our results showed that the OC values in diabetic rats not treated with VAC were significantly lower than that of baseline in normal rats, and similar results were obtained in previous reports [38,43,47]. After treatment with VAC, the plasma OC levels of the diabetic rats were obviously increased, although the plasma OC level of diabetic rats receiving VAC still appeared to be lower than the baseline in normal rats, suggesting that VAC may stimulate bone formation in diabetic rats. The plasma OC values in normal rats receiving VAC maintained the baseline values, as did those of normal control rats (not receiving VAC), indicating that VAC did not increase the osteoblast activity of normal rats. Unlike the decreased plasma OC levels in diabetic rats not treated with VAC, the plasma ALP levels in diabetic rats not receiving VAC were considerably increased compared with those in normal control rats (not receiving VAC), and the ALP values in diabetic rats treated with VAC remained at higher levels although vanadium compounds could partially inhibit ALP activity in fraction preparations from the osteoblasts [31], indicating that VAC may not affect in vivo ALP value. The elevation of plasma ALP may have been caused by (1) a fatty liver in diabetes and a depressed deactivation effect of the liver on ALP derived from the malnourished hepatopathy [48], or (2) enhanced activity of intestinal mucosa brush-border enzymes [49]. This increase in plasma ALP levels in diabetic rats was consistent with the reported data [50]. Nevertheless, a decrease in tibial ALP was observed in that study, suggesting that the increased ALP level in the plasma alone may not be sufficient to represent increased activity of osteoblasts. Tibial ALP values were not measured in the present study. In addition, our results showed that TRACP values were obviously increased in diabetic rats compared with those in normal rats, and were not affected by the VAC treatment, suggesting that VAC seemed not to inhibit osteoclast activity.

The present study revealed that VAC treatment did not impair the functions or the structures of the kidney and liver. These findings were consistent with those reported by Dai et al. [15] for a 1-year toxicity study of vanadyl sulfate.

It has also been reported [51–53] that prolonged administration of vanadium derivatives in animals caused hemolysis and impairment in erythropoiesis as well as in erythrocyte maturation, which were indicated by a decrease in the erythrocyte count and hemoglobin level, and an increase in the leukocyte count. The decrease in the erythrocyte count and increase in the leukocyte count and the mild depression of the hemoglobin level observed in our investigation were in agreement with previous results, suggesting that VAC treatment is slightly toxic to the blood system. The reversal of this slight impairment in the blood system after the withdrawal of VAC deserves further investigation.

After the administration of VAC to diabetic rats for 35 days, plasma glucose, CHOL, TRIG, and CRE were markedly improved, indicating that the diabetic state had improved. Furthermore, the decreased ultimate strength, Tb.Th, MAR, and plasma OC in diabetic rats were improved or normalized. Meanwhile, VAC did not affect any bone parameters in normal rats. These improvements to diabetic rat bone may be due to the improvement in the diabetic state.

In conclusion, VAC did not cause obvious signs of diarrhea, deaths, or any changes in the kidneys and liver in any groups. VAC treatment improved or normalized the abnormal bone parameters in diabetic rats, including ultimate strength, Tb.Th, MAR, and plasma OC values, but seemed not to affect the BMD, bone stiffness, bone energy absorption, O.Th, Tb.Sp, plasma ALP, and TRACP values in diabetic rats. VAC did not alter any bone parameters evaluated in normal rats. The results indicate that oral VAC is tolerated and benefits the diabetic osteopathy of rats, but seems

not to influence the bone of normal rats. They also suggest that VAC improves diabetes-related bone disorders, primarily by improving the diabetic state.

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