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The Vitamin D Receptor Activator Maxacalcitol Provides Cardioprotective Effects in Diabetes Mellitus

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Running title: Effects of Vitamin D on cardiac damage in diabetes

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Abstract

Purpose

Recent reports showed a significant association between vitamin D levels and cardiovascular disease events and mortality. In the current study, we investigated the effect of the vitamin D receptor activator maxacalcitol (OCT) on cardiac damage in a rat model of type 2 diabetes.

Methods

At 20 weeks of age, the rats were divided into three groups: vehicle-treated (DM), insulin-treated (INS) and OCT-treated (OCT). At 30 weeks, the rats were sacrificed and urinary and blood biochemical analyses and cardiac histological and immunohistochemical analyses were performed. To evaluate the effect of OCT on the renin-angiotensin system, we performed a further study using aliskiren (ALS). At 20 weeks, the diabetic rats were divided into two groups: the ALS-treated group (ALS) and the ALS plus OCT-treated group (ALS+OCT), and we evaluated the renin-angiotensin system (RAS) and cardiac lesions at 30 weeks.

Results

At 30 weeks, despite comparable blood pressure and renal function, heart volume, intracardiac oxidative stress by immunohistological analysis, cardiac and perivascular fibrosis and urinary excretion of 8-hydroxydeoxyguanosine and serum N-terminal pro-brain natriuretic peptide levels were significantly decreased in the OCT group compared to the DM group. mRNA expressions of dihydronicotinamide adenine dinucleotide phosphate (NADPH) p47 subunit and cardiac injury-related markers in the heart were also significantly decreased in the OCT group compared to the DM group. The cardioprotective effect of OCT was preserved even in the context of RAS inhibition.

Conclusion

Our results suggest that OCT prevents the development of cardiac damage in DM, independent of RAS inhibition.

Keywords: diabetes mellitus, oxidative stress, renin angiotensin system, vitamin D receptor activator

Introduction

Chronic kidney disease (CKD) is a major risk factor associated with cardiovascular events and mortality [1, 2]. Recently, the close association between CKD and cardiovascular disease (CVD) has become the focus of investigation. However, the pathophysiological mechanisms underlying this link are very complex and remain unclear. Renin-angiotensin system (RAS) inhibitors including angiotensin converting enzyme inhibitors, angiotensin receptor blockers and aldosterone blockers, not only delay the decline of kidney function and reduce urinary protein excretion but also prevent the progression of cardiac damage [3-5]. Furthermore, many basic and clinical reports demonstrated that β -blockers could have cardioprotective effects [6-8].

Recently, several studies have reported the organ-protective effects of vitamin D [9]. Meta-analyses and large cohort studies have demonstrated a significant association between vitamin D deficiency and negative CKD and CVD outcomes [10, 11]. Therefore, vitamin D may have an important role in preventing CKD and CVD complications. Furthermore, clinical and experimental studies have shown that vitamin D treatment reduces proteinuria, improves endothelial dysfunction, and ameliorates cardiac hypertrophy [12-15]. Among the causes of CKD, diabetes mellitus (DM) is also a crucial risk factor for CVD. It has been demonstrated that vitamin D deficiency is

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3 associated with DM [16, 17]. Considering these facts, we supposed that vitamin D
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6 therapy may improve the prognosis of patients with diabetes.
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10 VDRA is routinely used for dialysis patients to prevent the progression of secondary
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12 hyperparathyroidism in the clinical setting. In general, VDRA has lower affinity for
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14 vitamin D binding protein than vitamin D (25-hydroxyvitamin D and
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16 1,25-dehydroxyvitamin D). Therefore, VDRA is promptly excreted into urine from the
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18 kidney and its half-life is shorter than that of vitamin D. Furthermore, VDRA is easy to
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20 move to every tissue and exert its physiological action for a relatively short time.
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22 Because of its properties, VDRA does not so often manifest adverse effects such as
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24 vascular calcification and hypercalcemia compared to active vitamin D.
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35 Although there are several studies demonstrating the effect of vitamin D and VDRA
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37 on cardiac structure and function in CKD, to our knowledge, there is little data detailing
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39 the role of vitamin D and VDRA in diabetes. In the present study, we examined the
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41 effects of VDRA on the heart and the pathophysiological mechanisms underlying these
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43 effects on oxidative stress and RAS in a model rat of diabetes.
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54 **Materials and Methods**

55 ***Animals***

Male spontaneously diabetic Torii (SDT) rats were obtained from CLEA Japan Inc., Tokyo, Japan. The rats were housed with food and water available *ad libitum* in light- and temperature-controlled environments. At 20 weeks of age, these rats were divided into three groups: vehicle-treated (DM, $n=8$), insulin-treated (INS, $n=8$), and maxacalcitol-treated (OCT, $n=8$). Insulin pellets (LinShin Canada Inc., Scarborough, Ontario, Canada), which release a controlled amount of insulin, were subcutaneously implanted every two weeks. Maxacalcitol (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) was dissolved in phosphate-buffered saline (pH 8.0) containing 0.2% ethanol and 0.01% Tween 20, and administered intraperitoneally three times a week at a dosage of 0.2 $\mu\text{g/kg}$ body weight/day. A 24-h urine sample was collected from each rat using a metabolic cage before the rat was sacrificed. At 30 weeks, the rats were sacrificed under ether anesthesia. Blood samples for serum measurements were collected from the left ventricle, and the hearts were removed for RNA extraction and histomorphological analysis.

Furthermore, to perform additional analyses, we used aliskiren (ALS, Novartis, Switzerland), a direct renin inhibitor. Twenty weeks-old male SDT rats were divided into two groups: an ALS-treated group (ALS, $n=6$) and an ALS plus OCT-treated group (ALS+OCT, $n=6$). ALS was orally administered at a dosage of 100 mg/kg

bodyweight/day through a feeding tube. At 30 weeks, these rats were also sacrificed under ether anesthesia and evaluated.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee guidelines of Kobe University School of Medicine (Permit Number: P081214R). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Serum and urine measurements

After centrifugation for 5 min at 3000 rpm, serum samples obtained were stored at -80°C until analysis. Urine samples were also stored at -80°C for later analysis. Serum creatinine, total protein (TP), albumin (Alb), calcium (Ca) and phosphate (P) levels were measured using a FUJI DRI-CHEM 3500 (FUJIFILM Japan, Tokyo, Japan). Urinary creatinine levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Company, Ann Arbor, MI, USA). Urinary excretion of Alb (U-Alb) and 8-hydroxydeoxyguanosine (U-8-OHdG) were determined using an ELISA kit (Alb: Exocell Inc., Philadelphia, PA; 8-OHdG: Japan Institute for Control of Aging, Shizuoka, Japan). Serum N-terminal pro-brain natriuretic peptide (NT-proBNP)

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3 levels were also determined (Biomedica, Wien, Austria). Serum parathyroid hormone
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6 (PTH) levels were measured using a rat PTH-ELISA kit (Immutopics, San Clemente,
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9 CA, USA). Serum fibroblast growth factor 23 (FGF23) levels were measured using an
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12 intact FGF23 ELISA kit (Kainos Laboratories, Inc., Tokyo, Japan). Serum
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15 25-hydroxyvitamin D (25D) and 1,25-dihydroxyvitamin D (1,25D) levels were
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18 measured using a 25-Hydroxyvitamin D ¹²⁵I radioimmunoassay kit (DiaSorin Inc.,
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21 Stillwater, USA) and a TFB 1,25-dihydroxyvitamin D radioimmunoassay kit
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24 (Immunodiagnostic Systems Ltd., Boldon, UK), respectively.
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32 ***Blood pressure measurements***

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35 Systolic blood pressure measurements were performed by tail-cuff plethysmography
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38 (Model MK-2000; Muromachi Kikai Co. Ltd., Japan). To avoid the possibility of stress
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41 artefacts, the rats were allowed to acclimatize sufficiently. Blood pressure was
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44 measured at the basal time point, and then, every two weeks for the rest of the study
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47 period and determined using 10 readings for each rats.
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54 ***Histological and immunohistochemical analyses***

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57 To evaluate cardiac abnormalities, the hearts were removed, weighed and fixed with
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3 10% formaldehyde, dehydrated at room temperature through an ethanol series, and
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6 embedded in paraffin. The resulting paraffin blocks were cut into in 3- μ m-thick sections.
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9 The sections were stained with haematoxylin–eosin for routine histology and azan for
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12 morphometric studies. Myocardial fibrosis was evaluated in 20 randomly selected
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15 microscopic fields per sample using image analysis software - Lumina Vision version
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18 3.7.4.2 (Mitani Corp., Tokyo, Japan). As previously described [18], perivascular
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21 fibrosis was graded semiquantitatively on a scale of 1 to 4 (fibrosis score; normal to
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24 severe) in 20 random microscopic fields.
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28 The formation of 8-OHdG in the cardiac tissue was determined using anti-8-OHdG
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31 monoclonal antibodies (NOF Corporation, Tokyo, Japan). For the analysis, the heart
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34 slices were pre-incubated with blocking agents (Simple Stain Rat; Nichirei, Tokyo,
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37 Japan), followed by incubation with the primary antibodies mentioned above for 60 min
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40 at room temperature. Immune staining was performed using a universal
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43 immunoperoxidase polymer (Histofine Simple Stain MAX PO, anti-rat and anti-rabbit;
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46 Nichirei, Tokyo, Japan). The number of 8-OHdG-positive cells was counted in 20
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49 random microscopic fields per cardiac tissue section to calculate the 8-OHdG-positive
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52 cells scores.
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RNA extraction and real-time RT-PCR analyses

Total RNA was extracted from the heart using the ISOGEN kit (Wako Pure Chemicals Industries, Ltd, Osaka, Japan) as described previously [19]. Real-time PCR was performed using a LightCycler 350s Real-Time PCR System (Roche, Mannheim, Germany) with the LightCycler FastStart DNA Master SYBR Green I Kit (Roche). We analyzed all the data with the second derivative maximum method of the LightCycler software (version. 4.0; Roche). The relative mRNA expression levels of target genes were normalized to β -actin mRNA. Primer sequences used in this study are listed in Table 1.

Statistical analysis

We performed all statistical analyses using the computer software application StatView 5.0 (SAS Institute, Cary, NC, USA). Values are presented as means $\pm$ SEM. To evaluate the significance of differences between groups, Student's t-test or one-way ANOVA followed by the Tukey–Kramer test was used. A p value of < 0.05 was considered statistically significant.

Results

Animal characteristics and biochemical data

At 20 weeks of age, there were no significant differences in animal characteristics among the three groups (data not shown). As shown in Table 1, at 30 weeks of age, body weight, blood pressure, renal function and glycemic control were comparable between the DM and OCT groups. U-Alb was significantly less in the OCT group than in the DM group. In the INS group, body weight and serum TP and Alb levels were significantly greater, and HbA1c and U-Alb were significantly lower compared to the DM and OCT groups. As for the renal function, creatinine clearance (Ccr) tended to be lower in the INS group compared to that in the other two groups although it was not statistically significant. As for CKD-mineral bone disorder parameters, serum Ca, P, and 25D levels were comparable among the three groups. By contrast, serum 1,25D and PTH levels were significantly suppressed by OCT administration and these levels were lower in the DM group compared to the INS group. Serum FGF23 levels significantly increased following OCT administration and were the lowest in the DM group.

Change of cardiac findings by the OCT treatment

As shown in Figure 1A, at 30 weeks, the relative heart volume was significantly lower in the OCT group than in the DM group despite similar blood pressures in the two

groups. Serum NT-proBNP levels were also significantly lower in the OCT group than in the DM group (Figure 1B). The degree of myocardial and perivascular fibrosis was more severe in the DM group than in the INS and OCT groups (Figure 2A-D). The OCT treatment prevented the progression of cardiac fibrosis.

Assessment of oxidative stress

We examined systemic and intracardiac oxidative stress among the three groups. Twenty-four-hour U-8-OHdG was greater in the DM and OCT groups than in the INS group. U-8-OHdG tended to be lower in the OCT group than in the DM group although there was no statistically significant difference (Figure 3B). The 8-OHdG-positive cell score in the heart was significantly lower in the OCT group than in the DM group (Figure 3C).

mRNA expression of markers of cardiac hypertrophy

To assess the expressions of cardiac hypertrophy-related genes, the mRNA expressions of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), myosin heavy chain beta (β -MHC), myosin heavy chain alpha (α -MHC), connective tissue growth factor (CTGF) and dihydronicotinamide adenine dinucleotide phosphate

(NADPH) p47-phox subunit were determined by real time RT-PCR. At 30 weeks, the mRNA expressions of ANP, BNP, β -MHC/ α -MHC, CTGF and NADPH p47 phox were significantly higher in the DM group than in the INS group, and these increased expressions were reduced by OCT treatment (Figure 4).

Assessment of RAS

To evaluate RAS status, we measured the urinary excretion of angiotensinogen (AGT) and the mRNA expression of renin and angiotensin II type 1 receptor (AT1R). Urinary excretion of AGT was markedly elevated in the DM group, and significantly lower in the INS group than in the DM group. It did not significantly differ between the OCT group and the DM group (Figure 5). As for the mRNA expression of renin and AT1-R, although they were significantly lower in the INS group than in the DM and OCT groups, there was no significant difference between the DM and OCT groups (Figure 6).

Effect of OCT on heart under RAS inhibition

We performed a further study to ascertain whether OCT could prevent the progression of diabetic cardiomyopathy under RAS inhibition. As shown in figure 7,

OCT could attenuate cardiac hypertrophy in the context of RAS inhibition. However, the mRNA expression of RAS genes and the urinary excretion of AGT were comparable between the ALS group and the ALS+OCT group (Figure 8).

Discussion

Our study demonstrated that 1) at 30 weeks, despite comparable blood pressure and renal function, U-Alb and U-8-OHdG were significantly lower in the OCT group than in the DM group; 2) heart volume and serum NT-proBNP levels were significantly lower in the OCT group compared to the DM group; 3) the number of 8-OHdG positive cardiomyocytes was reduced and cardiac and perivascular fibrosis was ameliorated by OCT administration; 4) the mRNA expressions of cardiac injury-related markers were significantly decreased in the hearts of OCT-treated rats compared to the DM group; and 5) the cardioprotective effect of OCT was preserved in the context of RAS inhibition.

Numerous reports have indicated that vitamin D and VDRA have various beneficial effects on physiological functions [9, 12, 13]; moreover, previous experimental and clinical studies have also demonstrated the favourable effects of vitamin D and VDRA on cardiac lesions, including left ventricular hypertrophy (LVH) [14, 15, 20]. On the

other hand, randomized controlled trials in patients with CKD could not demonstrate the beneficial effects of VDRA on LVH [21, 22]. However, these studies also showed that the number of patients with cardiovascular-related hospitalizations was significantly lower in the VDRA treated group compared to the placebo group. Based on these results, we speculated that vitamin D and VDRA therapy may be effective for appropriate patients. It has been reported that vitamin D deficiency is prevalent among patients with type 2 diabetes and is associated with cardiac abnormalities [23]. Thus, in the present study we assessed the effect of VDRA therapy on cardiac abnormalities in diabetes.

CVD is the most important complication that is closely linked with mortality in diabetes. Although the rapid progression of coronary artery disease (CAD) is well known in diabetes, some patients with diabetes have prominent cardiac dysfunction and LVH despite having no significant CAD. This cardiac abnormality, now termed ‘diabetic cardiomyopathy’, occurs in diabetes independently of CAD and hypertension [24]. Several mechanisms responsible for diabetic cardiomyopathy have been proposed. Oxidative stress, activation of RAS, impaired regulation of intracellular calcium, abnormal cellular metabolism and mitochondrial dysfunction etc. seem to be involved in the progression of diabetic cardiomyopathy [25, 26].

Oxidative stress is one of the most important factors for the progression of CVD and CKD. Oxidative stress is remarkably increased in diabetes and plays a key role in the progression of various diabetic complications such as osteopenia, nephropathy and cardiac and vascular lesions. It has been demonstrated that vitamin D, including OCT, has anti-oxidative effects [27, 28]. In the present study, U-8-OHdG increased and increased oxidative stress in the heart was proven by immunohistochemical staining. These results showed that systemic and local oxidative stress was increased under diabetic conditions. Although glycemic control by insulin therapy could reduce oxidative stress and prevent the progression of organ damage, OCT therapy also decreased oxidative stress and allayed organ damage compared to the DM group despite the comparably high glucose levels. Therefore, we believe that OCT exerts anti-oxidative effects independently of glycemic control. The results of our study also revealed a significant decrease in heart volume as well as a reduced the expression of cardiac hypertrophy-related genes and cardiac and intracardiac vascular fibrosis, following OCT therapy, which is in line with previous studies under non-diabetic conditions. In the light of these results, OCT is considered to have cardioprotective effects in diabetic conditions.

To explain the favourable effects of vitamin D and VDRA on the cardiovascular system, several pathophysiological mechanisms have been speculated. Active vitamin D halts the progression of atherosclerosis by enriching Th2 lymphocytes population [29]. The improvement of endothelial dysfunction by vitamin D therapy has been also reported [12, 30]. In addition to these vascular protective effects, the suppression of RAS is thought to be one of the most important mechanisms accounting for the cardioprotective effects of VDRA [14]. However, a previous study has demonstrated that OCT does not suppress renin and angiotensin II [31]. In fact, our data also showed that there were no significant differences in the expressions of RAS-related genes in the heart and urinary excretion of AGT between the DM and OCT group. Furthermore, to ascertain whether the cardioprotective effect of OCT depends on the suppression of RAS, we performed a further study using aliskiren. Although there was no significant difference in oxidative stress between the ALS and ALS+OCT group, heart weight and expression of cardiac hypertrophy-related genes were significantly lower in the ALS+OCT group compared to those in the ALS group. Taken together, we suggest that OCT has a cardioprotective effect that is independent of RAS suppression and this effect is especially associated with the cardiac hypertrophy progression pathway. The calcineurin–nuclear factor of activated T cells (calcineurin-NFAT) signaling cascade

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3 seems to be a crucial pathway of pathological hypertrophy [32-34]. It has been reported
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6 that vitamin D could prevent the progression of cardiac hypertrophy through the
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9 inhibition of this pathway [35]. Although we could not prove the mechanisms in this
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12 study, inhibition of this pathway by vitamin D may be one of the mechanisms of
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15 reducing cardiac hypertrophy.
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19 Increased serum levels of FGF23 and PTH have been reported to be associated with
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22 cardiac hypertrophy [36-39]. In addition, impaired regulation of intracellular Ca leads to
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25 diastolic and systolic dysfunction [40, 41]. In the present study, although the details
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28 were not evaluated, the heart volume was the lowest in the INS group despite the
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31 highest serum PTH level among the three groups, and the heart volume in the OCT
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34 group was lower than in the DM group despite the highest serum FGF23 levels. These
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37 results suggest that FGF23 and PTH did not play a key role in our study. Furthermore,
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40 because an elevation of serum Ca levels following the administration of OCT was not
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43 observed, impaired Ca homeostasis did not seem to affect cardiac abnormalities.
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48 It has been reported that serum FGF23 levels are significantly associated with CVD
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51 events and mortality in both dialysis and non-dialysis patients [42, 43]. Certainly,
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54 though active vitamin D and VDRA raise FGF23 levels, they also increase the
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57 expression of klotho which has cardiovascular protective effects. Then, the previous
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3 experimental study has reported that not a slightly and moderately but extremely high
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6 concentration of FGF administration lead to cardiac hypertrophy [36]. Wolf has
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9 speculated that a degree of FGF response to vitamin D may be important and patients
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12 who exhibit a greater increase in FGF23 may manifest poor prognosis [44]. Our data
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15 also showed that elevation of serum FGF23 levels was not associated with cardiac
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18 hypertrophy and damage. Therefore, we think that the balance between favorable effects
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21 and adverse effects is important in using vitamin D and VDRA. Taken together, in the
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24 real world, vitamin D and VDRA treatment may be beneficial for most CKD patients.
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31 **Conclusions**

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35 Our data suggest that OCT prevents the development of cardiac damage, such as
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38 cardiac hypertrophy and cardiac fibrosis in diabetes mellitus, independently of RAS
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41 inhibition. To explore the detailed mechanisms, a further study is needed.
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47 **Acknowledgement:**

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51 This study was partly presented at the annual meeting of American Society of
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54 Nephrology Congress, 2013.
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Table 1. The primers used in real time RT-PCR amplification

Gene	Forward (5'→3')	Reverse (5'→3')
ANP	AAATCCCGTATACAGTGCGG	GGAGGCATGACCTCATCTTC
BNP	CCAGAACAATCCACGATGC	TCGAAGTCTCTCCTGGATCC
α -MHC	GAGCAGGAGCTGATCGAGAC	CCTCTGCGTTCCTACACTCC
β -MHC	CTCCAGAAGAGAAGAACTCC	CCACCTGCTGGACATTCTGC
CTGF	GCGGCGAGTCCTTCAA	CCACGGCCCCATCCA
NADPH p47 phox	CCGGTGAGATCCACACAGAA	TGCACGCTGCCCATCAT
Renin	CTGTGCATACTGGCTCTCCA	GGCTTGGCCTAAACTAGGG
AT1R	CTCAAGCCTGTCTACGAAAATGAG	GTGAATGGTCCTTTGGTCGT
β -actin	CGTCATCCATGGCGAACT	CCC GCGAGTACAACCTTCT

ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; α -MHC, alfa myosin heavy chain; β -MHC, beta myosin heavy chain; CTGF, connective tissue growth factor; AT1R, angiotensin II type 1 receptor.

Table 2. Animal Characteristics at 30 weeks of age

	DM (n=8)	INS (n=8)	OCT (n=8)
Body weight (g)	437.9±6.6 *	576.9±10.2	452.8±5.4 #
SBP (mmHg)	145.7±1.2 *	136.6±1.7	143.2±2.0
TP (mg/dL)	5.6±0.1 *	6.3±0.1	5.6±0.1 #
Alb (mg/dL)	3.4±0.1	3.6±0.1	3.3±0.1 #
HbA1c (%)	11.3±0.1 *	4.7±0.5	10.9±0.1 #
Ccr (ml/min)	9.7±0.5	7.2±0.6	9.0±1.3
U-Alb (mg/day)	74.3±7.7 *	14.6±1.7	36.6±2.7 # †
Ca (mg/dL)	11.1±0.2	10.8±0.2	10.8±0.3
P (mg/dL)	9.0±0.4	8.4±0.5	9.4±0.6
25 D (ng/mL)	18.4±0.9	18.5±0.9	17.0±0.7
1,25 D (ng/mL)	71.8±5.7 *	188.1±16.8	30.2±1.8 # †
PTH (ng/mL)	98.1±23.0	170.3±62.0	37.0±7.7 #
FGF23 (ng/mL)	451.7±49.6	633.8±104.1	1138.8±151.1 # †

SBP, systolic blood pressure; TP, total protein; Alb, albumin; HbA1c, haemoglobin A1c; Ccr, creatinine clearance; u-protein, 24 hour- urinary protein excretion; Ca, calcium; P, phosphate; 25D, 25-hydroxyvitamin D; 1,25D, 1,25-dehydroxyvitamin D; PTH, parathyroid hormone; FGF23, fibroblast growth factor 23.

* ; DM v.s INS, $p<0.05$, # ; OCT v.s INS, $p<0.05$, † ; DM v.s OCT, $p<0.05$

Figure Legends

Figure 1. Cardiac parameters in the DM, INS and OCT groups

(A) Heart volume

(B) Serum N-terminal pro-brain natriuretic peptide (NT-proBNP) levels

* ; DM group v.s INS group, $p<0.05$, † ; DM group v.s OCT group, $p<0.05$.

Figure 2. Histological examination of the heart sections in the DM, INS and OCT groups

(A) Myocardial fibrosis (magnification $\times 400$)

(B) Perivascular fibrosis (magnification $\times 400$)

(C) Degree of myocardial fibrosis

(D) Perivascular fibrosis score

* ; DM group v.s INS group, $p<0.05$, † ; DM group v.s OCT group, $p<0.05$.

Figure 3. Immunohistochemical evaluation of oxidative stress in the cardiac tissue in the DM, INS and OCT groups.

(A) Left panel, DM group; middle panel, INS group; right panel, OCT group.

(B) Urinary 8-hydroxy-2'-deoxyguanosine (8-OHdG) excretion in the DM, INS and OCT groups.

(C) 8-OHdG-positive cell score in the heart ($\times 400$).

* ; DM group v.s INS group, $p < 0.05$, † ; DM group v.s OCT group, $p < 0.05$, # ; DM group v.s OCT group, $p < 0.05$.

Figure 4. mRNA expressions of cardiac hypertrophy-related genes in the DM, INS and OCT groups.

(A) mRNA expression of atrial natriuretic peptide (ANP)

(B) mRNA expression of brain natriuretic peptide (BNP)

(C) mRNA expression of beta-myosin heavy chain/alpha-myosin heavy chain (β -MHC/ α -MHC)

(D) mRNA expression of connective tissue growth factor (CTGF)

(E) mRNA expression of NADPH p47

* ; DM group v.s INS group, $p<0.05$, † ; DM group v.s OCT group, $p<0.05$, # ; DM group v.s OCT group, $p<0.05$.

Figure 5. mRNA expressions of renin-angiotensin system-related genes and urinary excretion of angiotensinogen (AGT) in the DM, INS and OCT groups.

(A) mRNA expression of renin

(B) mRNA expression of angiotensin II type 1 receptor (AT1R)

(C) 24hr-urinary excretion of AGT

* ; DM group v.s INS group, $p<0.05$, † ; DM group v.s OCT group, $p<0.05$, # ; DM group v.s OCT group, $p<0.05$.

Figure 6. Heart weight between the ALS and ALS+OCT groups.

; ALS group v.s ALS+OCT group, $p<0.05$.

Figure 7. mRNA expressions of cardiac hypertrophy-related genes between the ALS and ALS+OCT groups.

(A) mRNA expression of atrial natriuretic peptide (ANP)

(B) mRNA expression of brain natriuretic peptide (BNP)

(C) mRNA expression of beta-myosin heavy chain/alpha-myosin heavy chain (β -MHC/ α -MHC)

(D) mRNA expression of connective tissue growth factor (CTGF)

(E) mRNA expression of NADPH p47

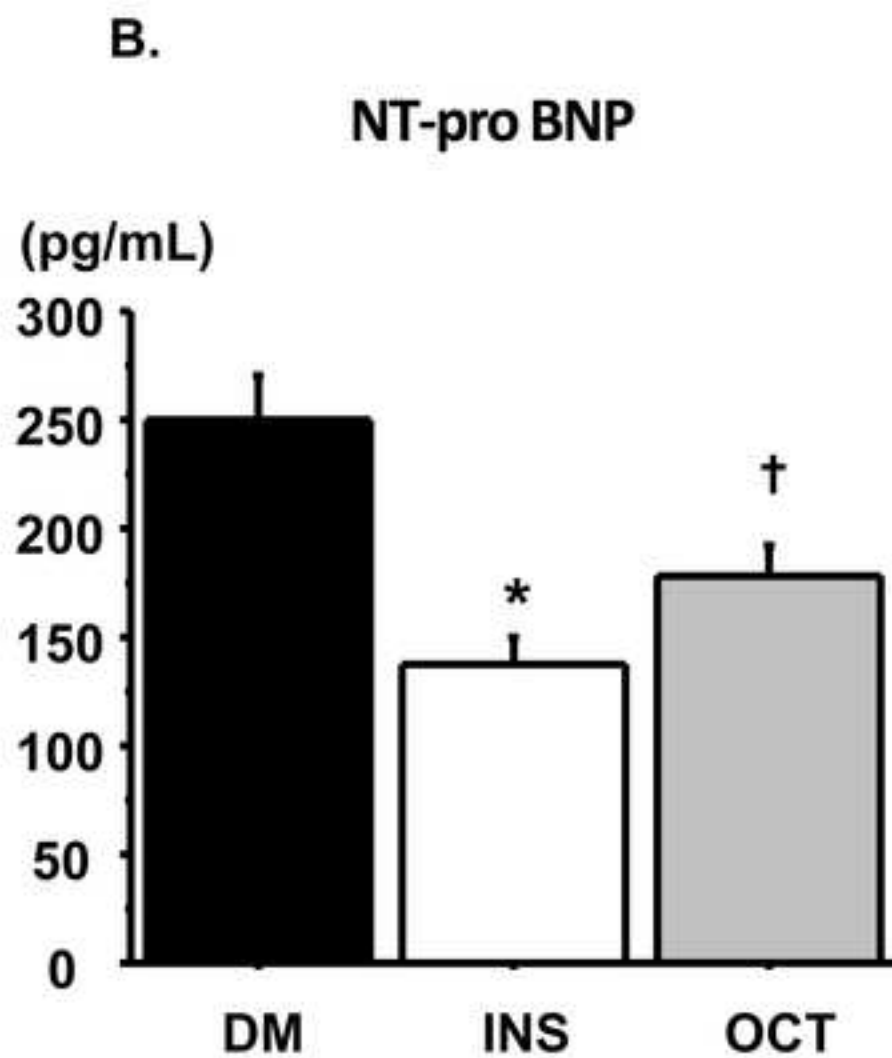
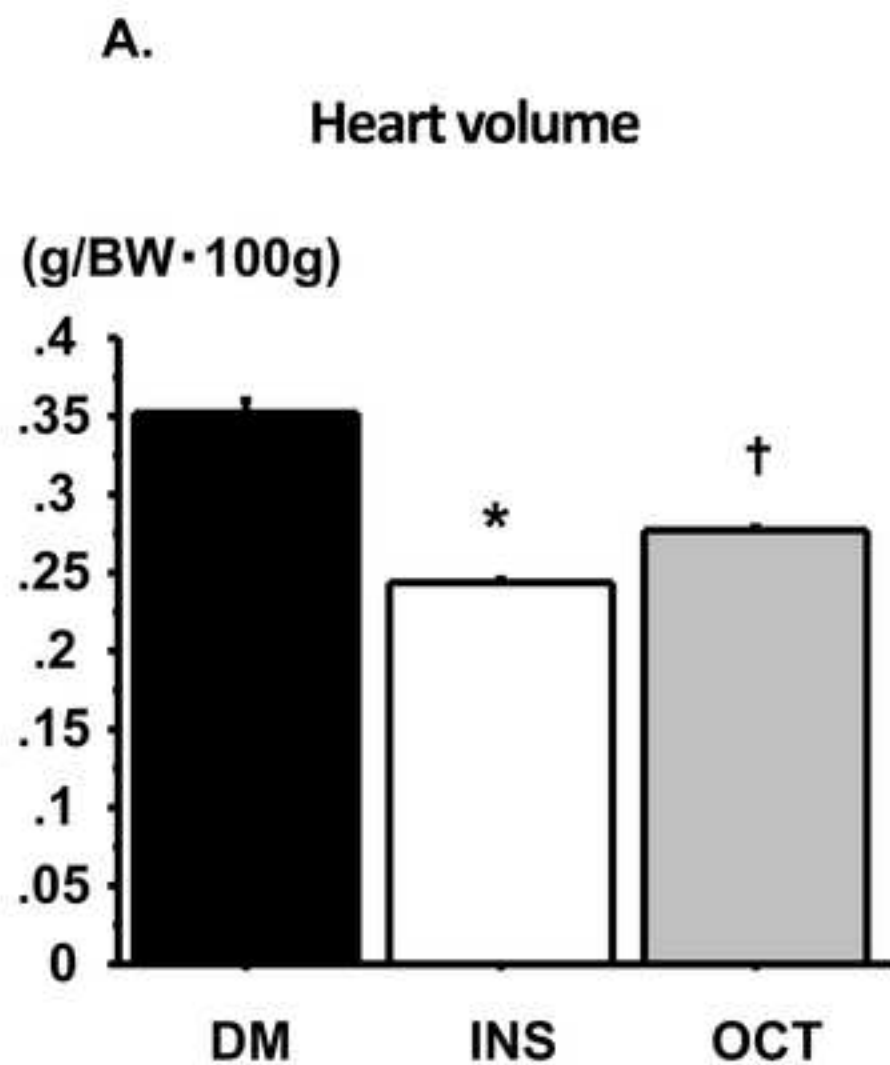
; ALS group v.s ALS+OCT group, $p < 0.05$.

Figure 8. mRNA expressions of renin-angiotensin system-related gene and urinary excretion of angiotensinogen in the ALS and ALS+OCT groups.

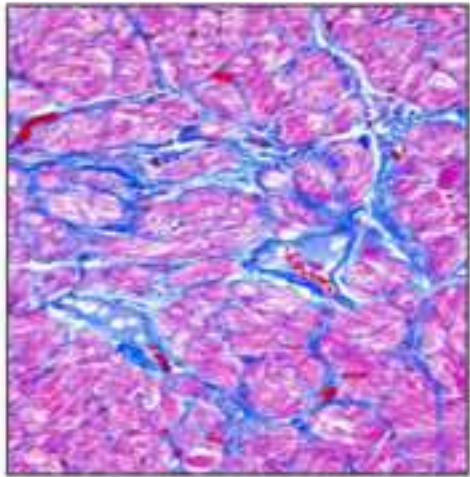
(A) mRNA expression of renin

(B) mRNA expression of angiotensin II type 1 receptor (AT1R)

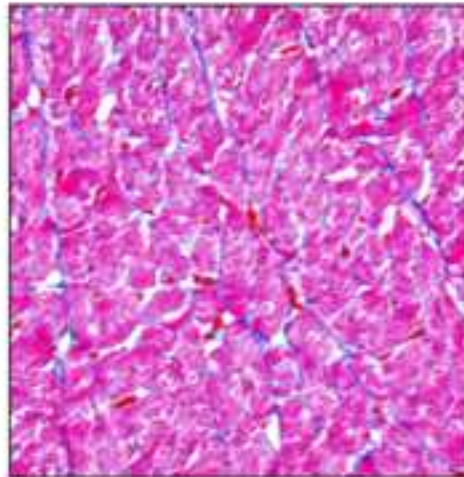
(C) 24hr-urinary excretion of angiotensinogen (AGT)



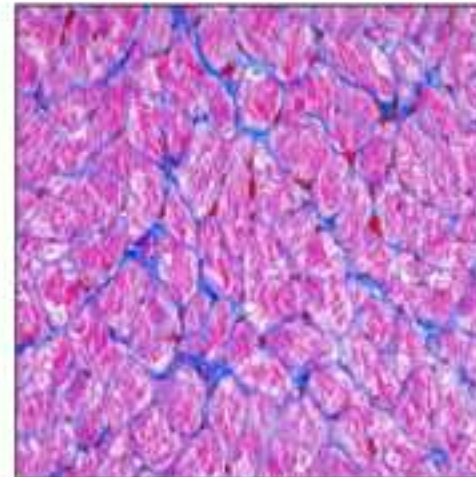
A. Cardiomyocyte (× 400)



DM

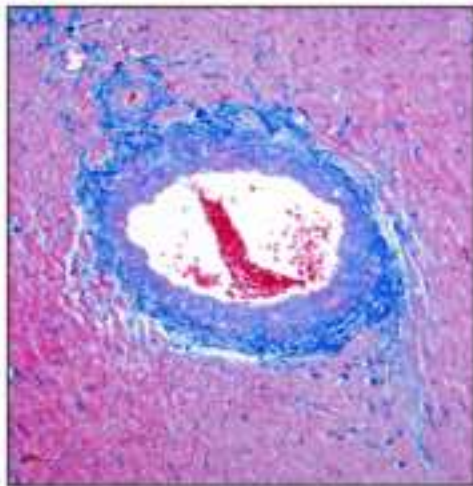


DM + INS

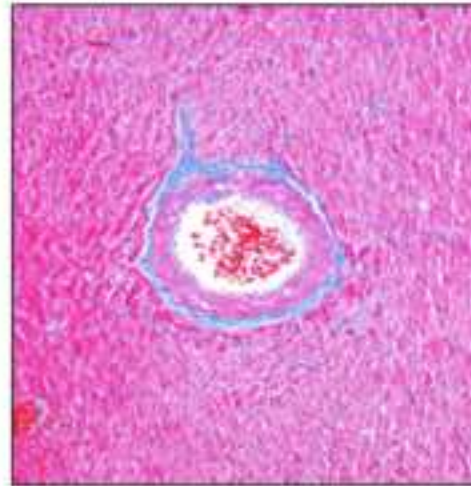


OCT

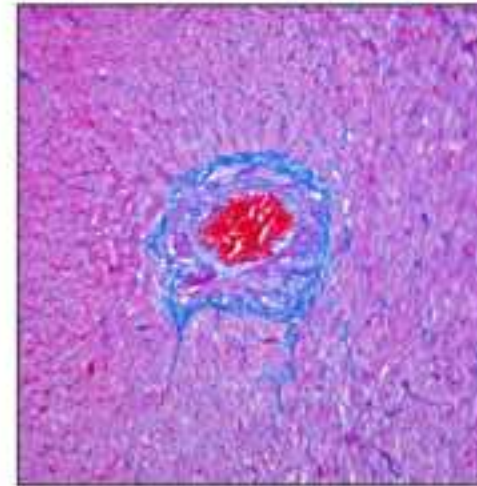
B. Perivascular (× 400)



DM



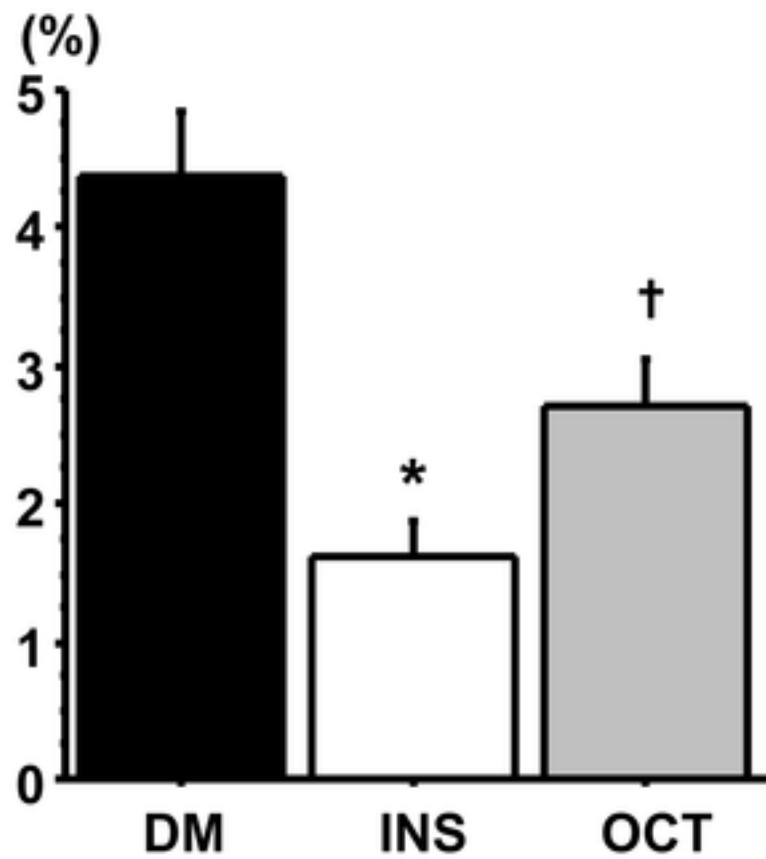
DM + INS



OCT

C.

Cardiac fibrosis



D.

Perivascular fibrosis

