Effect of Vitamin D Analogues on Acute Cardiorenal Syndrome: A Laboratory Rat Model

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ABSTRACT: **Background:** Vitamin D has been shown to induce beneficial effects on cardiovascular and renal morbidity by regulating inflammation and tissue fibrosis.

Objectives: To evaluate the effect of vitamin D analogues on cardiac function and fibrosis in an animal model of cardiorenal syndrome.

Methods: Unilateral nephrectomy was performed and myocardial infarction induced in rats. The rats were treated with vitamin D receptor activator (VDRA, paricalcitol, 40 ng/250 g x 3/week) versus a vehicle. A third group of animals, which served as the control, underwent sham surgery and received no treatment. After 4 weeks of treatment, cardiac function and fibrosis were assessed by trans-thoracic echo and histology, respectively. As a parameter of systemic inflammation, previously shown to be altered in acute coronary syndrome, T regulatory (Treg) cell levels were measured by flow cytometry. Renal dysfunction was documented by standard laboratory tests.

Results: After 4 weeks of treatment, no significant improvement in cardiac function parameters was noted following VDRA administration. VDRA treatment did not significantly alter Treg cell systemic levels. Consistently, despite a trend toward less extent of myocardial fibrosis, we found no clear beneficial effects of VDRA on myocardial tissue inflammation and remodeling.

Conclusions: Vitamin D treatment showed no beneficial effects on cardiac function parameters and fibrosis in an animal model of cardiorenal syndrome.

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KEY WORDS: vitamin D analogues, cardiorenal syndrome (CRS), myocardial fibrosis, cardiac function, T regulatory cells (Treg)

An intriguing interaction, termed cardiorenal syndrome, occurs between the heart and the kidney if the function of either organ is compromised. The effect of renal impairment on cardiovascular function has been demonstrated in various studies [1-4]. Impaired renal function correlates directly with cardiovascular morbidity, which remains the major cause of death among these patients [3,5]. In parallel, Hillege and co-authors [6,7] found that renal dysfunction is a stronger predictor of mortality than impaired cardiac function in advanced chronic heart failure.

Myocardial infarction may lead to impaired renal function as shown by an increase in serum creatinine levels and proteinuria [8]. Both mechanisms are thought to induce a vicious cycle of cardio-renal interaction, which may account for the increased morbidity and mortality in patients with cardiovascular and renal diseases. These interactions were investigated in both clinical and experimental studies. The models hypothesized to explain the described relationships include hemodynamic alterations, involvement of the renin-angiotensin-aldosterone system and the sympathetic nervous system, endothelial dysfunction, reactive oxygen species, and inflammation [9-14].

Accumulating data show that vitamin D exerts potential beneficial effects on cardiovascular and renal disease in patients with chronic kidney disease [15-18]. This phenomenon occurs independent of a mineral metabolism effect but rather relies on immunomodulation, reduced inflammation and retardation of fibrosis. The vitamin D receptor was previously shown to be expressed on cardiac myocytes [19,20]. Mizobuchi et al. [21] demonstrated that vitamin D receptor activators suppressed the development of left ventricular hypertrophy as well as myocardial and perivascular fibrosis in a CKD rat model. Unfortunately, the clinical benefits of these observed effects are limited in light of the abundant use of vitamin D in CKD patients.

Using a rat model of cardiorenal syndrome, we sought to assess whether vitamin D would improve cardiac function and tissue fibrosis after acute MI in animals with moderate renal impairment.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS

The local animal experimental committee approved all protocols. Male Lewis rats weighing 300–350 g (n=35) (Harlan, Israel) were housed under standard conditions with free access to food and drinking water. Rats received a normal salt diet throughout the study.

CKD = chronic kidney disease
MI = myocardial infarction

RAT MODEL OF CRS: SURGICAL PROCEDURES

Laparotomy was performed under anesthesia with ketamine. The right kidney was separated from the adrenal gland and the surrounding tissue. The renal artery and vein, as well as the urethra, were ligated with a 4.0 silk suture and cut, followed by removal of the right kidney (uninephrectomy). Rats were allowed to recover from surgery in proper conditions. One week after nephrectomy, rats were intubated, connected to a ventilator (Amsterdam Infant Ventilator, Hoek/Loos, Schiedam, The Netherlands) and anesthetized by administration of 3% isoflurane in N_2/O_2 (2:1). Left-sided thoracotomy was performed and the left coronary artery was occluded 2 to 5 mm from its origin with a 6.0 silk suture. The wound was closed, anesthetics were shut off, and 100% oxygen was administered until the rat was able to breathe normally.

STUDY GROUPS

There were three study groups. Group 1 comprised rats with UnX + MI and treatment with intraperitoneal injections of paricalcitol (Zemplar®), a vitamin D synthetic analogue (4 ng/250 g) 3 times a week starting 24 hours after MI (n=15); group 2 comprised rats with UnX + MI and treatment with vehicle 3 times week starting 24 hours after MI (n=15); and group 3 was the sham (control) group, comprising rats with laparotomy + thoracotomy without UnX or MI and without any treatment (n=5). Treatments lasted 4 weeks.

FUNCTIONAL PARAMETERS

Heart function tests were evaluated after 4 weeks of treatment by trans-thoracic echo using the Acuson XP 10 cardiac ultrasound machine with a 15 MHz transducer that has 128 imaging elements configured in a phased-array format. Measurements of heart rate, ejection fraction, fractional shortening, left ventricular end-systolic and end-diastolic volumes were measured. All animals were held in a metabolic cage (Lavotal, Italy) for 24 hours. Urine collection for Cr clearance and NGAL measurement was taken. CCT was calculated using the following formula: [Cr (urine)/Cr (serum)*urine volume]/1440. Urine NGAL levels were determined by enzyme-linked immunosorbent assay according to the manufacturers' instructions (Bioporto Diagnostics, Denmark, for Ngal) using the same urine samples collected for the CCT calculation.

AUTOPSY

Four weeks after MI induction, all animals were sacrificed using a CO_2 chamber. Laparotomy was performed and blood was drained through the vena cava for Cr readouts. Hearts, remaining kidneys and spleens were removed, flushed and weighed.

UnX = uniphrectomy
Cr = creatinine
NGAL = neutrophil gelatinase-associated lipocalin
CTT = creatinine clearance

HISTOLOGY

Cardiac ventricles were dissected from the atria and large vessels, and the right free wall (right ventricle) was separated from the left ventricle. A left ventricular mid-sagittal slice (approximately 2 mm) was fixed in Bouin solution, embedded in paraffin, and cut into 5 µm slices which were stained with hematoxylin & eosin. A pathologist blinded for the groups evaluated all sections.

EVALUATION OF REGULATORY T CELL LEVELS

Splenocytes were suspended in phosphate-buffered saline (106/100 µl) and stained with anti-mouse CD4-FITC and anti-mouse CD25-PE (Miltenyi, Germany) for 30 minutes at 4°C, washed and resuspended in 0. 5 ml fixation buffer for 1 hour at 4°C and then washed twice with permeabilization buffer (E-Bioscience, USA). Next, $8x10^4$ cells were acquired by flow cytometry and the percentage of Treg cells from the total CD4+ cells was calculated.

STATISTICAL ANALYSES

Differences between groups were compared using a two-tailed *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

GROSS OBSERVATIONS

Compared with the control group, animals with cardiorenal syndrome showed a decline in total body weight [Figure 1A]. No significant difference between groups was noted regarding VDRA-treated versus vehicle-treated animals. Heart weight-to-total body weight ratio showed an incline in CRS animals,

Treg = regulatory T cells
VDRA = vitamin D receptor activators
CRS = cardiorenal syndrome

Figure 1. Effect of vitamin D on gross parameters. **[A]** Total weight differences between treated and untreated animals. **[B]** Differences in heart weight to total body weight ratio between treated and untreated animals. VDRA = vitamin D analogue-treated animals, VEH = vehicle-treated animals, CON = control group, WT = weight

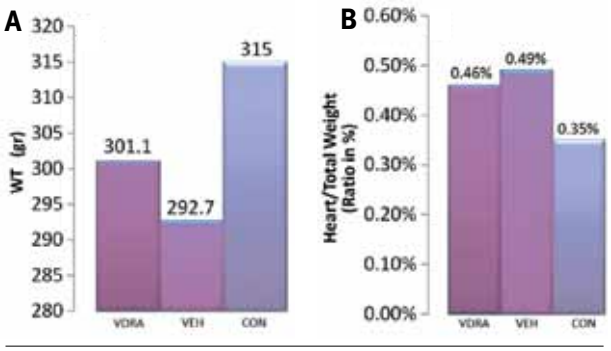
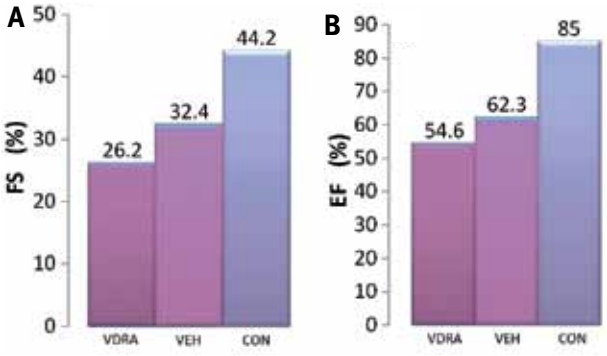


Figure 2. Comparison of heart function parameters between vitamin D-treated (VDRA) and untreated animals: **[A]** fractional shortening (FS) and **[B]** ejection fraction (EF). VEH = vehicle-treated animals, CON = control group



which concurs with an elevation in heart size [Figure 1B]. The VDRA-treated animals did not show a significant decline in that parameter.

CARDIAC AND RENAL FUNCTION

Fractional shortening and ejection fraction showed a significant reduction in CRS animals, but no benefit was noted with VDRA treatment compared to vehicle treatment [Figure 2A and B]. An elevation in blood urea nitrogen and Cr levels was noted in CRS animals, with no statistical significant reduction between the VDRA- and the vehicle-treated groups [Figure 3A and B]. Notably, urine NGAL levels were higher in the post-MI animals. A slight reduction was shown in the VDRA group but with no statistical significance [Figure 3C].

T REGULATORY CELL NUMBER

Treg cells have an immunomodulatory function in inflammatory states. Their number was shown to incline after an MI [22]. In our study, Treg numbers were higher in the CRS groups, but treatment with VDRA did not result in a significant decrease/increase in their numbers [Figure 3D].

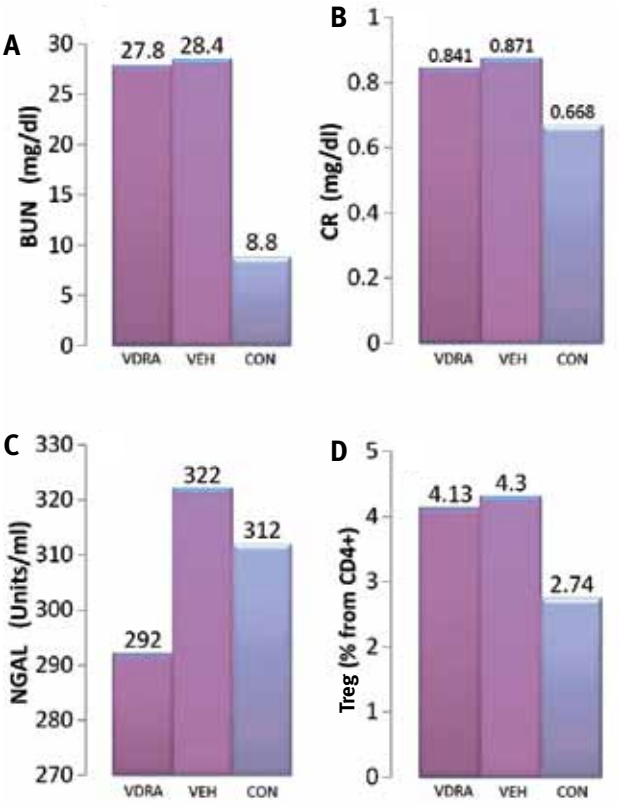
HISTOLOGY

Infarction was demonstrated in the heart tissue of all the CRS animals. Fibrosis and disturbance to the myocardial muscle structure distal to the infarcted area was shown in one rat from the VDRA group and three rats from the vehicle group [Figure 4A and B].

DISCUSSION

The relationship between vitamin D and coronary artery disease was established in several studies in the past several years. Based on a cohort of over 27,000 subjects, Bair et al.

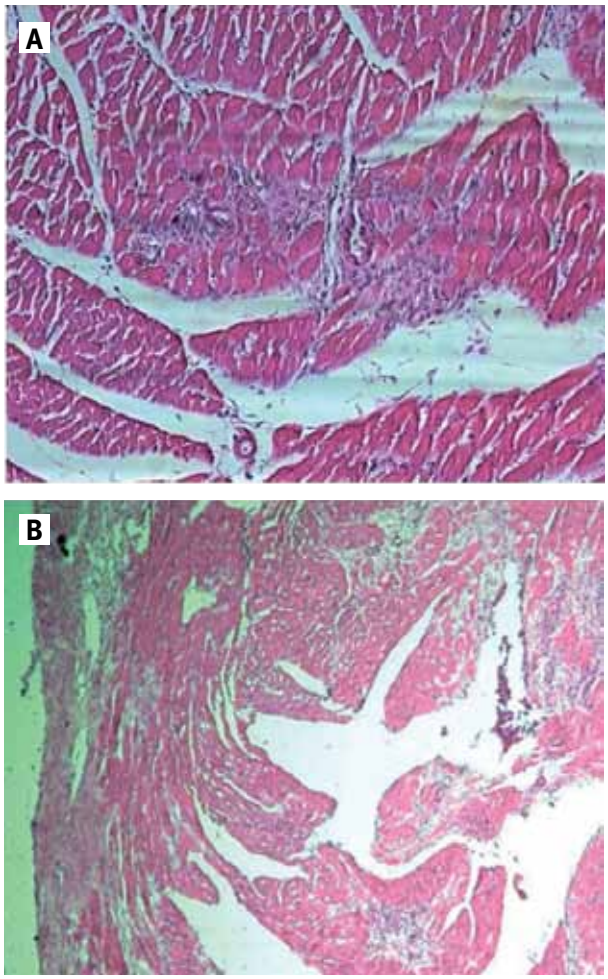
Figure 3. Comparison of kidney function parameters between vitamin D-treated (VDRA) and untreated animals: **[A]** serum blood urea nitrogen (BUN) levels, **[B]** serum creatinine (Cr) levels, **[C]** urine NGAL levels, and **[D]** Treg counts. VEH = vehicle-treated animals, CON = control group, NGAL = neutrophil gelatinase associated lipocalin, Treg = T regulatory cells



[22] found a correlation between vitamin D deficiency and increased risk for various heart diseases including heart failure, coronary artery disease and atrial fibrillation. The Framingham Offspring study showed an increased risk for first cardiovascular event in conjunction with decreased levels of vitamin D [23]. Furthermore, Oh and colleagues [24] demonstrated that vitamin D ameliorated foam cell formation in the atherosclerotic plaque in diabetic patients, and VDRA was found to decrease left ventricular and coronary artery fibrosis in uremic laboratory animals [21].

To our knowledge, our study is the first to question the effect of vitamin D analogs in the acute phase of post-MI myocardial injury. In our experimental rat model no significant clinical or laboratory benefits were demonstrated. As expected, a substantial reduction in heart function was noted in the CRS animals but no statistically significant improvement was shown in the VDRA-treated group. Interestingly, total body weight was lower in the post-MI animals, while

Figure 4. Fibrosis foci distal from the infarct zone in vehicle-treated animals



it would be expected that salt and fluid accumulation would result in increased body weight. A possible explanation is that heart failure resulted in malabsorption and thus in failure to thrive in the young rats tested in our experiment.

Renal function deterioration, as reflected in serum and urine tests, did not show an improvement after VDRA treatment. As previously discussed, Treg cells play an important role in maintaining immunological unresponsiveness to self-antigens and in suppressing excessive immune and inflammatory responses deleterious to the host [25]. The anti-inflammatory effect of vitamin D was demonstrated in previous studies [25]. Consequently, we tested the effect of VDRA on the population of Tregs in acute CRS. Yet, no statistically significant reduction in Tregs was noted. Nevertheless, examination of pathology slides demonstrated that focal fibrosis distal to the infarcted area was less prevalent in the VDRA-treated group. As previously discussed, focal lesions of fibrosis not in conjunction with the infarct zone were shown in three of seven non-VDRA-treated animals and in one of eight VDRA-treated animals. These findings are consistent with previous studies that showed the positive effect of vitamin D on retardation of fibrosis [21].

There are several possible explanations for our findings. As in previous studies, the sample size of the animal groups in this study was relatively small, with numbers ranging from 10 to 15. With such a sample size it is difficult to demonstrate subtle effects that might have been shown had the groups been larger. Furthermore, surveillance time for the study was only 4 weeks. In such a short period, important histological alterations may not translate fully into laboratory and clinical results.

In summary, despite a trend toward less extent of myocardial fibrosis, no clear beneficial effects of vitamin D analogs on cardiac function were noted in animals with acute CRS. Whether vitamin D analog should be administrated in these patients remains unanswered and requires further investigation.

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Capsule

Mucin protein MUC2 as guardian of the gut

The intestine is able to tolerate continual exposure to large amounts of commensal bacteria and foreign food antigens without triggering an inappropriate inflammatory immune response. In the large intestine, this immunological tolerance is thought to occur via a physical separation between environment and host imposed by a continuous mucous layer built up from the secreted mucin protein, MUC2. However, in the small intestine, this mucous layer is porous, necessitating an additional layer of immune control. Shan et al. report that in the small intestine, MUC2 plays an active role in immunological tolerance by activating a transcription factor in resident dendritic cells, thereby selectively blocking their ability to launch an inflammatory response. This work identifies MUC2 as a central mediator of immune tolerance to maintain homeostasis in the gut and possibly on other mucosal surfaces in the body.

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Capsule

Immune clearance of highly pathogenic SIV infection

Established infections with the human and simian immunodeficiency viruses (HIV and SIV, respectively) are thought to be permanent with even the most effective immune responses and antiretroviral therapies only able to control, but not clear, these infections. Whether the residual virus that maintains these infections is vulnerable to clearance is a question of central importance for the future management of millions of HIV-infected individuals. Hansen and team recently reported that approximately 50% of rhesus macaques (RM; *Macaca mulatta*) vaccinated with SIV protein-expressing rhesus cytomegalovirus (RhCMV/SIV) vectors manifest durable, aviremic control of infection with the highly pathogenic strain SIVmac239. They show that regardless of the route of challenge, RhCMV/SIV vector-elicited immune responses control SIVmac239 after demonstrable lymphatic and hematogenous viral dissemination, and that replication-competent SIV persists in several sites for weeks to months. Over time, however, protected RM lost signs of SIV infection, showing a consistent lack of measurable plasma- or tissue-associated virus using ultrasensitive assays, and a loss of T cell reactivity to SIV determinants not in the vaccine. Extensive ultrasensitive quantitative polymerase chain reaction (PCR) and quantitative PCR with reverse transcription analyses of tissues from RhCMV/SIV vector-protected RM necropsied 69–172 weeks after challenge did not detect SIV RNA or DNA sequences above background levels, and replication-competent SIV was not detected in these RM by extensive co-culture analysis of tissues or by adoptive transfer of 60 million hematolymphoid cells to naive RM. These data provide compelling evidence for progressive clearance of a pathogenic lentiviral infection, and suggest that some lentiviral reservoirs may be susceptible to the continuous effector memory T cell-mediated immune surveillance elicited and maintained by cytomegalovirus vectors.

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