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Vitamin D attenuates HMGB1-mediated neointimal hyperplasia after percutaneous coronary intervention in swine

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Abstract

Aims: Intracoronary stenting is a common procedure in patients with coronary artery disease (CAD). Stent deployment stretches and denudes the endothelial layer, promoting a local inflammatory response, resulting in neointimal hyperplasia. Vitamin D deficiency associates with CAD. In this study, we examined the association of vitamin D status with high mobility group box 1 (HMGB1)-mediated pathways (HMGB1, receptor for advanced glycation end products [RAGE], and Toll-like receptor-2 and -4 [TLR2 and TLR4]) in neointimal hyperplasia in atherosclerotic swine following bare metal stenting.

Methods and Results: Yucatan microswine fed with a high cholesterol diet were stratified to receive vitamin D-deficient (VD-DEF), vitamin D-sufficient (VD-SUF), and vitamin D-supplemented (VD-SUP) diet. After 6 months, PTCA (percutaneous transluminal balloon angioplasty) followed by bare metal stent implantation was performed in the left anterior descending (LAD) artery of each swine. Four months following coronary intervention, angiogram and optical coherence tomography (OCT) were performed and swine euthanized. Histology and immunohistochemistry were performed in excised LAD to evaluate the expression of HMGB1,

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RAGE, TLR2 and TLR4. OCT analysis revealed the greatest in-stent restenosis area in the LAD of VD-DEF compared to VD-SUF or VD-SUP swine. The protein expression of HMGB1, RAGE, TLR2, and TLR4 was significantly higher in the LAD of VD-DEF compared to VD-SUF or VD-SUP swine.

Conclusion: Vitamin D deficiency was associated with both increased in-stent restenosis and increased HMGB1-mediated inflammation noted in coronary arteries following intravascular stenting. Inversely, Vitamin D supplementation was associated with both a decrease in this inflammatory profile and in neointimal hyperplasia, warranting further investigation for Vitamin D as a potential adjunct therapy following coronary intervention.

Article Summary:

We analyzed the association of Vitamin D status on HMGB1-mediated pathways in coronary arteries after the deployment of bare metal stent in an atherosclerotic swine model. Our study has identified a significant degree of neointimal hyperplasia after percutaneous coronary angioplasty among vitamin D-deficient swine compared with sufficient or supplemented vitamin D status, indicating translational significance with vitamin D supplementation after coronary intervention.

Keywords

Toll like receptors; High mobility group box1; Vitamin D; Intimal hyperplasia; Restenosis; Intravascular stenting

1. Introduction

Coronary artery disease (CAD) remains a major cause of morbidity and mortality in the developed world [1]. While the advent of percutaneous coronary transluminal angioplasty (PTCA) has been a viable approach to revascularization, increased incidence of restenosis in 30-50% of patients within 3-5 years remains a major drawback [2-3]. Furthermore, while the additional use of bare metal stents (BMS) or drug-eluting stents (DES) in the coronary arteries has substantially improved the patency rates, their utilization has been burdened by hypersensitivity, in-stent restenosis (ISR) and the risk of late thrombosis [4-6]. Secondary to post-interventional endothelial denudation and sub-endothelial matrix exposure, neointimal hyperplasia (NIH) is the central underlying cause of restenosis [7]. Due to mechanical injury to the endothelium, NIH is an inflammatory process that involves uncontrolled proliferation of the vascular smooth muscle cells (VSMCs) and migration to the intima layer with the activation of fibroblasts leading to deposition of extracellular matrix (ECM) and reduction in the luminal area of the vessel [8].

Vitamin D is a potent immunomodulator and via activation of its receptor (vitamin D Receptors [VDRs]) on many immune cells, it generates many anti-inflammatory and defensin molecules to combat inflammatory response [9-10]. Indeed, the active form of vitamin D, 1,25(OH)₂D₃, inhibits the production of pro-inflammatory cytokines via both the inhibition of IL-12 production and upregulation of dendritic cell (DC)-IL-10 to induce the generation of regulatory T-cells [11-13]. Low serum levels of vitamin D have been associated with increased risk of CAD [14]. Within coronary arteries of an atherosclerotic

swine model, it has been shown that NIH following PTCA is associated with decreased expression of VDRs in proliferating VSMCs and that vitamin D supplementation may exhibit protective effects against NIH [15-16].

Vitamin D is a fat-soluble vitamin and a steroid hormone that exhibits multiple roles, including calcium-phosphorous homeostasis, skeletal health, and regulation of immunity with anti-inflammatory effects [17]. In the realm of cardiovascular disease (CVD), low levels of vitamin D have been associated with increased risk of myocardial infarction, cardiac hypertrophy, cardiomyopathy, heart failure, cardiac fibrosis, and rhythm abnormalities, in addition to CAD [18]. While the possibility of confounding factors in these studies exist, evidence from the National Health and Nutrition Examination III (NHANES III) follow-up study showed that high vitamin D levels are independently and inversely associated to CAD and all-cause mortality [19]. Furthermore, in patients with carotid stenosis, low intraplaque VDR expression with prevalence of M1 macrophages (pro-inflammatory) vs M2 macrophages (anti-inflammatory) was noted to predict major adverse cardiovascular events (MACEs) [20]. While vitamin D has also been shown to alter macrophage polarization and cholesterol efflux to limit NIH and post-interventional coronary restenosis in an atherosclerotic swine model, the underlying mechanisms and further anti-inflammatory relationships with NIH pathogenesis remain unclear and require further attention [15-16]. Further evaluation is required to investigate if vitamin D status is causally related to the inflammatory profile noted in both CAD and NIH.

Among the inflammatory profile associated with the development of atherosclerotic plaque, damage-associated molecular patterns (DAMPs) have been found to be released by stressed necrotic cells in the intima rendering plaques unstable [21]. HMGB1 is one of the major DAMPs that increases with the progression of atherosclerosis via the production of a series of inflammatory cytokines (interferon gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α), interleukin-1beta [IL-1 β], and interleukin-6 [IL-6]) [22]. Additionally, upregulation of HMGB1 is associated with an increased level of Toll-like receptors (TLRs), particularly TLR2 and TLR4, which have been associated as major factors in the pathogenesis of atherosclerosis and implicated in driving the vascular inflammatory process in NIH [23-25]. In addition to TLR2 and TLR4, HMGB1 is also a ligand for receptor for advanced glycation end products (RAGE), a multi-ligand pattern recognition receptor that potentiates the immediate innate inflammatory responses [26]. AGEs serve as ligands for RAGE and are believed to promote atherosclerotic plaque progression and instability via increased macrophage migration, increased lipid accumulation within macrophages and VSMC dysfunction, all secondary to AGE interaction with RAGE found on the plaque lesion [27-31]. Likewise, this AGE-RAGE interaction has been noted to play a central role with NIH, with the accumulation of AGE noted after arterial injury [32-33].

The goal of this study was to examine the association of vitamin D status with high mobility group box 1 (HMGB1)-mediated pathways of NIH in coronary arteries after the deployment of bare metal stenting in an atherosclerotic swine model.

2. Materials and Methods

2.1. Swine Model, Vitamin D-regulated Diet, and embedding of stented coronary artery

Yucatan™ miniature female swine were purchased from Sinclair Bio-resources (Windham, MA) and were randomized into three experimental groups, according to the status of vitamin D, consisting of three animals in each group with similar body weights between 30-40 lbs. We used female micropigs since they are less aggressive, safer and easier to handle than males. Also, the commercially available male pigs are castrated for safety of the personnel, but castration induces hormonal changes which may be a confounding factor in cardiovascular studies. The swine were fed with an atherogenic diet purchased from Teklad (Envigo, USA). Animal work was performed in accordance with the guidelines of the National Institutes of Health and USDA for the care and use of experimental animals.

Creighton University Institutional Animal Care and Use Committee approved the animal research protocol (#0831.2). Animals were housed free from sunlight in a secured Animal Resource Facility at Creighton University.

Three different diets were selected, and animals stratified to receive these diets. Three microswine were fed a mixture of Teklad swine diets 8753 and 10125 (Envigo, USA), which was designated as the vitamin D-deficient high cholesterol diet composed of 19% “Vitamin-Free” Test Casein (to precisely control vitamin D content), 23.5% sucrose, 23.9% corn starch, 13% maltodextrin, 4% soybean oil, 4% cholesterol, and 10% cellulose, with the addition of 0.55% sodium cholate and 9% chocolate. These microswine received 500 IU of vitamin D3/per day (Vitamin D-deficient diet) with food.

The six microswine were fed a mixture of Teklad swine diets 8753 and 9487 (Envigo, USA), a high cholesterol diet (HC) that consisted of 37.2% corn (8.5% protein), 23.5% soybean meal (44% protein), 5% alfalfa, 4% cholesterol, 4% peanut oil, 1.5% sodium cholate, and 1% lard; with the addition of 8% chocolate. These six microswine were equally divided into two groups: Vitamin D-sufficient diet group where 1,500 IU of vitamin D3/per day was administered (Vitamin D-sufficient diet), and vitamin D-supplemented diet group received 4,500-5,000 IU of vitamin D3/per day (vitamin D-supplemented diet).

The total glucose, protein and fat levels were similar in the diets of all three experimental groups. The only difference was in the amount of vitamin D in the diet.

Serum 25(OH)D levels were monitored at baseline, before coronary intervention and at euthanasia, from venous blood (10 ml) drawn from the auricular vein. Seventy-two hours prior to intervention, all swine were placed on an aspirin (350 mg/day) and ticlopidine (325 mg/day) regimen. Angioplasty with Bare Metal Stent (BMS) implantation was performed in the left anterior descending artery (LAD).

Animals were anesthetized by administering intramuscular (i.m.) injection of a single dose mixture of xylazine (2 mg/kg) and tiletamine-zolazepam (5 mg/kg, 1:1 mixture of tiletamine hydrochloride and zolazepam hydrochloride). The unconsciousness was confirmed by the lack of blinking response, jaw tone, and change in respiratory and heart rate. This was followed with inhaled isoflurane (3-4% isoflurane mixed with 2L/min oxygen using a face

mask). After 15 mins, endotracheal intubation was performed, and inhalational isoflurane (1-3% mixed with 2L/min oxygen using a face mask) was continued throughout the procedure. A prophylactic pre-operative one-time dose of cefazolin (3-5 mg/kg; i.m.) was administered. Intravenous catheters were placed in the auricular vein on both sides for normal saline (NS) and the administration of a prophylactic anti-arrhythmic drug (Amiodarone i.v. initially 4-5 mg/kg followed by 0.05 mg/kg/hr prior to stent insertion).

The groin was shaved and prepped in a sterile fashion with betadine and alcohol scrubs and prepped sterile. A 5-6 cm incision was made, femoral vessels were identified, and the femoral artery was punctured. A 4F sheath was introduced followed by 6F sheath to maintain the vascular access patent for insertion of cardiac catheters. Prophylactic administration of the anti-arrhythmic drug (Amiodarone i.v. initially 4-5 mg/kg followed by 0.05 mg/kg/hr) was started prior to stent insertion as mentioned and continued during the procedure. An angiography guiding catheter (6F AR2/100 cm) was advanced to the left coronary ostium and coronary angiography was performed by injecting a non-ionic contrast media and imaged using C arm (OEC 9900 Elite Vas 8, GE Healthcare).

After introducing a guide wire into the LAD, a cobalt chromium Bare Metal Stent of 2.5 mm x 15 mm (Abbott™) was advanced to reach the LAD and inflated to 10-15 atmospheres (ATMs) utilizing the inflation device with a pressure gauge (Basix Touch™). The balloon was subsequently deflated, and angiography performed to evaluate the patency of the artery. All catheters were removed, and the femoral artery puncture site was repaired using a 4-0 polypropylene (Prolene) sutures. The groin and skin incisions were closed with polyglycolic-acid (Vicryl) sutures. After completion of the procedure, the animal was extubated after return of reflexes.

During post-operative recovery, the animal was covered with a warm blanket and IV fluids administered until the swine was able to stand unassisted. A single dose of analgesic (buprenorphine; 0.01-0.03 mg/kg, i.m.) was given to relieve the pain which was continued every 12 hours for the first three days. During the first ten post-operative days, the animals were monitored daily for signs of pain or any other discomfort.

2.2. Optical Coherence Tomography (OCT) and Percent Restenosis

The frequency domain (FD)-OCT examination was performed at 16-24 weeks after stent insertion. Briefly, after intracoronary administration of nitroglycerin (100–300 µg), a 0.014-in standard guide wire was positioned distally to the stent. Subsequently, the FD-OCT catheter (Dragonfly™ DUO imaging catheter; St. Jude Medical, St. Paul, MN, USA) was advanced to the distal edge of the target region. Iodine contrast media was simultaneously injected during FD-OCT pullback. The entire region of interest was scanned at infusion rates ranging between 6-22 cm³ at 2–10 cm³/s and 350 psi. Quantitative FD-OCT of images was analyzed via Light Lab OCT imaging proprietary software (Light Lab Imaging). The percentage of neo-occlusion was calculated as follows: [(mean reference lumen area– minimum lumen area) ÷ mean reference lumen area] × 100. Neointimal area was calculated as Stent area – Lumen area.

None of the animals suffered any fatal or life-threatening events observed prior to being euthanized after the procedure. Euthanasia was performed by intravenous administration of a single dose of pentobarbital sodium (85 mg/kg) and phenytoin sodium (11 mg/kg). Swine were observed for absence of heartbeat and respiration for 5 minutes prior to tissue harvest. The heart was removed, and the coronary arteries were excised and processed in 10% formalin. The LAD stented vessels were processed in a Sakura Tissue Tek VIP Tissue Processor. Then the stented LAD arteries were infiltrated, polymerized and embedded with a Technovit® 9100 methyl methacrylate (MMA) kit following the manufacturers' instructions. Briefly, the stented LAD arteries were infiltrated with pre-infiltration solution (Technovit® 9100 methyl methacrylate basic solution with hardener) for 24 hours at 4°C, infiltrated with infiltration solution (Technovit® 9100 methyl methacrylate basic solution with hardener and PMMA powder) for 24 hours at 4°C, embedded with polymerization mixture for 10 minutes within a vacuum sealed desiccator at 4°C, removed from the vacuum desiccator and allowed to polymerize for at least 24 hours at -4°C. The polymerized tissue tubes were cut and shaped with a band saw to remove excess MMA and to embed the tissues into blocks. The tissue blocks were glued to block holders using Aron Alpha® Ethyl Ultra Speed Adhesive (Quickbond). Sections from the tissue block were cut with a tungsten carbide knife (Leica™, Germany) in a Leica RM2265 rotary microtome (Leica™, Germany) and attached to slides.

2.3. Immunohistochemistry

From each tissue, several thin sections (4-5 µm) were cut and used for immunostaining with different antibodies. We stained at least 3 adjacent sections from each tissue on separate slides. Tissue sections on the slides were de-plasticized, the sections were washed through solutions of 2-methoxyethylacetate for 30 min thrice, followed by acetone for 5 min twice, and distilled water for 5 min twice. Then rehydrated in ethanol and rinsed in double-distilled water. The slides were then treated with citrate buffer (AP-9003-500, Lab Vision™) in a steamer for 20 minutes. After a 20-minute cool down period, they were washed with phosphate buffered saline (PBS) and endogenous peroxidases were blocked in Bloxall™ (Vector laboratories) for 10 minutes and rinsed again in PBS. Blocking serum from a VECTASTAIN™ ABC kit was incubated on slides for 1 hour at room temp. Slides were drained and the tissue sections on the slides were incubated with the primary antibody from Santa Cruz Biotechnology® (anti-TLR4 sc293072, anti-TLR2 sc21760, anti-HMGB1 sc56698, anti-RAGE sc345154) at 4°C overnight. Slides were rinsed in 1xPBS, and then the tissue sections on the slides were exposed to the secondary antibody from the VECTASTAIN™ ABC kit for 1 hour at room temp. Slides were rinsed in 1xPBS, followed by incubation of the tissue sections with the VECTASTAIN™ ABC-HRP (horseradish peroxidase) for 30 minutes at room temp. Tissue sections on the slides were then rinsed with 1xPBS followed by addition of the Impact DAB (3,3'-diaminobenzidine) from Vector laboratories for 2 to 5 minutes until the brown color of the DAB started to form. After the DAB had developed sufficiently, slides were washed, stained in hematoxylin, rinsed, dehydrated in ethanol and cleared in xylene. Tissue sections were viewed with a Nikon Eclipse Ci microscope and images were photographed with a Nikon DS-L3 camera. These images were captured using the Nikon DS-L3 software. The 40x images from each tissue

sections were manually analyzed using cell counter from Image J and the average of the positive cell count from each group was used for statistical analysis.

2.3. Statistical analysis

From each immunostained slides, four areas were randomly selected in a clockwise manner under the microscope for the intensity analysis of immunostaining. The average of these intensities from each slide was taken into final quantitative analysis. Data are presented as the mean $\pm$ standard error of mean. Data were analyzed using GraphPad Prism 7.01. Multiple group comparisons were performed by Tukey's multiple comparison test using One-way ANOVA. Probability (P) value <0.05 was accepted as statistically significant.

3. Results

3.1 Vitamin D deficiency correlates with in-stent restenosis

Vitamin D status was confirmed by circulating levels of 25(OH)D. The baseline serum 25(OH)D levels measured prior to starting the experimental diets were 29 ± 1 ng/ml in vitamin D-deficient group, 34 ± 3 ng/ml in vitamin D-sufficient group, and 28 ± 2 ng/ml in vitamin D-supplemental group. After feeding the animals with vitamin D modified diets, the serum 25(OH)D levels at the time of euthanasia were measured. The levels of serum 25(OH)D among the vitamin D-deficient group was 7.0 ± 0.2 ng/ml, for the vitamin D-sufficient group was 39 ± 0.9 ng/ml and for the vitamin D-supplemented group was 82 ± 16 ng/ml. These values were maintained from the period of coronary intervention.

Optical coherence tomography (OCT) was performed just prior to euthanasia (see Figs. 1a-1c). Morphometric analyses of the entire proximal to distal end of the coronary arteries, where the stents were deployed, indicated significantly smaller lumen area (1.60 ± 0.26 mm²) in the vitamin D-deficient animals compared to the stented sites of vitamin D-sufficient (2.93 ± 0.13 mm²) and vitamin D-supplemented swine (3.43 ± 0.24 mm²).

OCT quantification of the stent area identified greater neointima formation at the interventional region of LADs in the vitamin D-deficient swine (3.24 ± 0.24 mm²) compared to the interventional region in the LADs of vitamin D-sufficient (2.33 ± 0.65 mm²) and vitamin D-supplemented swine (1.26 ± 0.35 mm²).

Thus, the degree of post-interventional percentage area in-stent restenosis was greatest in the stented LADs of the vitamin D-deficient swine ($67.7 \pm 5.12\%$) (Fig. 1a), compared to stented LADs of swine in the vitamin D-sufficient ($49.9 \pm 1.94\%$) (Fig. 1b) and vitamin D-supplemented group ($29.5 \pm 4.7\%$) (Fig. 1c). These individual mean differences were statistically significant among each other (Fig. 1g). Finally, the evaluation of percent restenosis/mm² within each stent yielded consistent measures for in-stent and edge-stent restenosis—indicating entire-stent restenosis as predicted by coronary injury model.

Histological characterization by H&E of resin-embedded stented LADs revealed results similar to OCT outcomes. Noticeably, sections of vitamin D-deficient stented LADs demonstrated an increased amount of neointimal hyperplasia ($65.6 \pm 4.94\%$) (Fig. 1d) compared to sections from vitamin D-sufficient ($49.9 \pm 2.19\%$) (Fig. 1e) and vitamin D-

supplemented ($29.5 \pm 2.37\%$) (Fig. 1f) stented LADs. These individual mean differences were statistically significant among each other (Fig. 1h).

3.2. Immunohistochemistry

3.2.1 Vitamin D deficiency is associated with increased expression of HMGB1 and RAGE—Immunohistochemistry was used to examine the relationship between circulating 25(OH)D levels and the post-interventional redistribution of HMGB1-positive and RAGE-positive cells (Fig. 2). Vitamin D-deficiency significantly upregulated the expression of HMGB1 (Fig. 2a) in stented LADs. The high intensity of HMGB1 immunopositive cells was prominent in the neointima, adventitia, and stent struts. In comparison to the LADs of vitamin D-deficient swine (105 ± 6 counts/HPF) (Fig. 2a), the LADs of swine fed with vitamin D-sufficient (56 ± 3 counts/HPF) ($p < 0.001$) (Fig. 2b) and vitamin D-supplemented (65 ± 5 counts/HPF) ($p < 0.001$) (Fig. 2c) diets demonstrated a decrease in HMGB1 expression.

In all coronary arteries, RAGE immunopositive cells were identified in the media, neointima and adventitia. Clusters of RAGE+ cells were located near stent struts in vitamin D-deficient and vitamin D-sufficient LAD (Fig. 2d-e). The density of RAGE+ cells were significantly more in the vitamin D-deficient (201 ± 65 counts/HPF) compared to vitamin D-sufficient (56 ± 5 counts/HPF) ($p < 0.01$) and vitamin D-supplemented groups (69 ± 4 counts/HPF) ($p < 0.01$) (Fig. 2h).

3.2.2 Vitamin D suppresses the expression of TLR2 and TLR4—Vitamin D deficiency was associated with an increased recruitment of HMGB1-mediated pathways, which also resulted in the increased recruitment of inflammatory cells through the evidenced increase in TLR2 and TLR4 (Fig. 3). Most of the cells in the media and neointima were immunopositive to TLR2 and TLR4. Cluster of cells surrounding stent struts were immunopositive to TLR4 (Fig. 3A) and TLR2 (Fig. 3d) in the vitamin D-deficient group. These positive clusters were not found around stent struts in vitamin D-sufficient and vitamin D-supplemented groups (Fig. 3b, 3c, 3e, 3f). The density of TLR4+ cells was significantly more in the vitamin D-deficient (89 ± 6 counts/HPF) compared to vitamin D-sufficient (56 ± 4 counts/HPF) ($p < 0.001$) and vitamin D-supplemented groups (41 ± 5 counts/HPF) ($p < 0.001$) (Fig. 3g). The density of TLR2+ cells was significantly more in the vitamin D-deficient (88 ± 30 counts/HPF) compared to vitamin D-sufficient (23 ± 2 counts/HPF) ($p < 0.01$) and vitamin D-supplemented groups (29 ± 3 counts/HPF) ($p < 0.02$) (Fig. 3h).

4. Discussion

The major findings in this study are, compared to a Vitamin D sufficient group: (1) Vitamin D deficiency was associated with increased NIH and that this extended to an observed increase in HMGB1-mediated inflammatory recruitment (via HMGB1, RAGE, TLR2 and TLR4) within post-interventional coronary arteries; and (2) *In-vivo* Vitamin D supplementation was inversely associated with NIH and with an observed decrease in HMGB1-mediated inflammatory recruitment after coronary intervention in an atherosclerotic swine model.

The development of atherosclerosis in swine by use of feeding a high fat diet has allowed for the creation of a well-established CAD model that can be closely related to post-angioplasty restenosis in human coronary arteries [34-35]. In our investigation as well, high serum lipid levels were noted to correspond to significant atherosclerotic plaque formation among swine that consumed a high cholesterol diet. Post-intervention via intravascular stenting did lead to the development of NIH and in-stent restenosis (ISR) in all coronary arteries, as appreciated by OCT which can provide high resolution to differentiate arterial layers. However, the greatest percentage of both ISR and NIH secondary to PCTA was most notable in the coronary arteries of vitamin D-deficient swine with a significantly increased neointimal thickness compared to vitamin D-sufficient or vitamin D-supplemented swine. Conversely, oral vitamin D supplementation, as evidenced by increased serum 25(OH)D levels, was associated with the greatest attenuation of NIH as noted by decreased neointimal thickness from OCT. This *in-vivo* anti-proliferative finding supports prior evidence supporting the hypothesis that vitamin D status is inversely correlated to the progression of NIH after coronary intervention. Additionally, the utilization of bare metal stents facilitated this understanding of vitamin D's response without the influence of the interference by drugs employed in drug-eluting stents in comparison.

With obligatory vessel injury with PTCA followed by intravascular stent placement, mechanical disruption of the native endothelium promotes VSMC proliferation, macrophage migration, and ECM deposition central to restenosis underlying the inflammatory process that is NIH.⁸ Among several inflammatory cytokines previously implicated with NIH, the stress response by necrotic intimal cells (DAMPs and its signaling) has been of interest. In this study, we found an increased inflammatory profile involving increased expression of HMGB1 receptor system (with TLR2 and TLR4) in the neointimal cells of post-interventional coronary arteries of vitamin D-deficient swine that decreased with increased vitamin D levels. In addition to the upregulation of pro-inflammatory cytokines, HMGB1 has previously been shown to induce VSMC proliferation and migration within human atherosclerotic plaques [8,36]. The connection of HMGB1 as potential activators of TLR2 and TLR4 was noted earlier, with the implication of TLRs with neointimal remodeling also shown and related to the ability of several TLRs to recognize DAMPs and induce sterile inflammation [37-39].

Recently, by genetic deletion and inhibition of its interaction with TLR4, HMGB1 inhibition was shown to prevent NIH after endoluminal arterial injury, implicating this ligand-receptor system directly connected with restenosis [20]. Within our study, we noted that the increased post-interventional NIH and ISR corresponded to an increase in both the neointimal expression and immunoreactivity to HMGB1 and both TLR2 and TLR4. Additionally, given the supporting evidence of pro-inflammatory relationship of HMGB1 and both TLR2 and TLR4 with post-interventional restenosis, the decreased presence and expression of these mediators with increasing vitamin D levels support the anti-inflammatory role of vitamin D and implicate DAMPs, among many cytokines, for further mechanistic inquiry. Of note, while direct mechanistic evidence of vitamin D's anti-proliferative capacity along HMGB1, RAGE, TLR2 and TLR4 within VSMCs is unknown, vitamin D has been shown to block the nuclear export of HMGB1 to the cytoplasm of macrophages responding to sepsis [40]. Considering its upstream regulatory activity of RAGE, TLR2 and TLR4, it can be speculated

that the downregulation of HMGB1 may then decrease the activity of these mediators as well. Furthermore, the mechanistic evidence of vitamin-D dependent suppression of endothelin-induced VSMC proliferation via inhibition of cell cycle machinery (e.g. cyclin dependent kinase 2) is also of interest with HMGB1 given evidence of HMGB1-induced cell cycle arrest [41-42].

While otherwise unclear, within asthmatic mice, vitamin D has been shown to reduce the inflammatory response through its interaction with HMGB1-TLR4 receptor signaling, with notable increases in serum IL-10 levels [43]. Furthermore, in this study, we have also demonstrated an increase in RAGE and its AGE ligands among this inflammatory profile that is increased with post-interventional NIH and ISR, as noted in the neointimal cells of stented and post-PTCA coronary arteries from vitamin D deficient swine and decreases with vitamin D status as well. Our current findings support previous findings that have noted the accumulation of RAGE and its ligands (AGE) within NIH after arterial injury [28-29].

Aside from the inflammatory milieu it promotes with atherosclerosis, within vascular injury the AGE-RAGE interaction has been noted to increase transforming growth factor-beta (TGF- β) to promote the necessary VSMC migration that underlies NIH [44]. However, the downregulation of RAGE-AGE by vitamin D is not clear, although serum levels of vitamin D have been shown to be negatively correlated to blood AGE levels [45]. Therefore, given the pro-inflammatory action of AGE-RAGE receptor system evidenced with NIH previously and in our study here, immunomodulatory effects of vitamin D noted here deserves to be explored in this context as well.

Study Limitations

The limitations in this study consist of not extending our investigation further into the underlying mechanisms that exist between vitamin D status and the observed modulation of HMGB1 mediated pathways. Additionally, the sole use of female microswine in our study does raise the possibility that our results could be influenced by the differential expression of sex hormones or of sex chromosome genes unknown to current knowledge. However, the ubiquitous cardioprotective effects of estrogen may have diminished the magnitude of the anti-inflammatory effect of vitamin D. Therefore, these investigations are certainly warranted in further studies.

5. Conclusions

In summary, we have found a significant degree of NIH after PTCA specially for In-stent restenosis associated with vitamin D-deficient swine compared with sufficient or supplemented vitamin D status. In addition to this finding, we also identified that vitamin D deficiency was significantly associated with increased HMGB1-mediated activity, which results in the increased recruitment of inflammatory cells through the evidenced increase in TLR2 and TLR4. Additionally, this also included intimal recruitment of RAGE and its ligand noted within vitamin D deficient swine. Likewise, vitamin D supplementation was associated with a suppression of this specific inflammatory activity and a diminish in NIH and restenosis after coronary intervention in this atherosclerotic swine model. Therefore,

vitamin D supplementation may prove to be a useful adjunct therapy to prevent post-intervention coronary restenosis.

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References

- [1]. Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. *PLoS Med* 2006; 3: e442. [PubMed: 17132052]
- [2]. Nobuyoshi M, Kimura T, Nosaka H, Mioka S, Ueno K, Yokoi H, et al. Restenosis after successful percutaneous transluminal coronary angioplasty: serial angiographic follow-up of 229 patients. *J Am Coll Cardiol* 1988; 12: 616–623. [PubMed: 2969925]
- [3]. Rupprecht HJ, Brennecke R, Bernhard G, Erbel R, Pop T, Meyer J. Analysis of risk factors for restenosis after PTCA. *Cathet Cardiovasc Diagn* 1990; 19: 151–159. [PubMed: 2317853]
- [4]. Buccheri D, Piraino D, Andolina G, Cortese B. Understanding and managing in-stent restenosis: a review of clinical data, from pathogenesis to treatment. *J Thorac Dis.* 2016; 8(10):E1150–E1162. [PubMed: 27867580]
- [5]. Joner M, Finn AV, Farb A, Mont EK, Kolodgie FD, Ladich E, et al. Pathology of drug-eluting stents in humans: delayed healing and late thrombotic risk. *J Am Coll Cardiol* 2006; 48: 193–202. [PubMed: 16814667]
- [6]. Nebeker JR, Virmani R, Bennett CL, Hoffman JM, Samore MH, Alvarez J, et al. Hypersensitivity cases associated with drug-eluting coronary stents: a review of available cases from the Research on Adverse Drug Events and Reports (RADAR) project. *J Am Coll Cardiol* 2006; 47: 175–181. [PubMed: 16386683]
- [7]. Yoshida Y, Mitsumata M, Ling G, et al. Migration of medial smooth muscle cells to the intima after balloon injury. *Atherosclerosis Ann N Y Acad Sci.* 1997; 811:459–470.
- [8]. Collins MJ, Li X, Lv W, et al. Therapeutic strategies to combat neointimal hyperplasia in vascular grafts. *Expert Rev Cardiovasc Ther.* 2012; 10(5):635–647 [PubMed: 22651839]
- [9]. Brennan A, Katz DR, Nunn JD, Barker S, Hewison M, Fraher LJ, et al. Dendritic cells from human tissues express receptors for the immunoregulatory vitamin D3 metabolite, dihydroxycholecalciferol. *Immunology* 1987; 61: 457–461. [PubMed: 2832307]
- [10]. Veldman CM, Cantorna MT, DeLuca HF. Expression of 1,25-dihydroxyvitamin D(3) receptor in the immune system. *Arch Biochem Biophys* 2000; 374: 334–338. [PubMed: 10666315]
- [11]. Lemire JM, Archer DC, Beck L, Spiegelberg HL. Immunosuppressive actions of 1,25-dihydroxyvitamin D3: preferential inhibition of Th1 functions. *J Nutr* 1995; 125: 1704S–1708S. [PubMed: 7782931]
- [12]. Penna G, Adorini L. 1 Alpha,25-dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J Immunol* 2000; 164: 2405–2411. [PubMed: 10679076]
- [13]. Canning MO, Grotenhuis K, de Wit H, Ruwhof C, Drexhage HA. 1-alpha,25-Dihydroxyvitamin D3 (1,25(OH)(2)D(3)) hampers the maturation of fully active immature dendritic cells from monocytes. *Eur J Endocrinol* 2001; 145: 351–357. [PubMed: 11517017]
- [14]. Chen S, Swier VJ, Boosani CS, Radwan MM, Agrawal DK. Vitamin D Deficiency Accelerates Coronary Artery Disease Progression in Swine. *Arterioscler Thromb Vasc Biol.* 2016; 36(8):1651–9. [PubMed: 27255724]
- [15]. Gupta GK, Agrawal T, Del Core MG, Hunter WJ 3rd, Agrawal DK. Decreased expression of vitamin D receptors in neointimal lesions following coronary artery angioplasty in atherosclerotic swine. *PLoS One* 2012; 7: e42789. doi:10.1371/journal.pone.0042789 [PubMed: 22880111]

- [16]. Gupta GK, Agrawal T, Rai V, Core MGD, Hunter WJ, Agrawal DK. Vitamin D Supplementation Reduces Intimal Hyperplasia and Restenosis following Coronary Intervention in Atherosclerotic Swine. *Plos One*. 2016;11(6). doi:10.1371/journal.pone.0156857.
- [17]. Holick MF. Vitamin D deficiency. *N Engl J Med*. 2007;357(3):266–81. [PubMed: 17634462]
- [18]. Rai V, Agrawal DK. Role of Vitamin D in Cardiovascular Diseases. *Endocrinology and Metabolism Clinics of North America*. 2017;46(4):1039–1059. doi:10.1016/j.ecl.2017.07.009. [PubMed: 29080634]
- [19]. Daraghme AH, Bertoia ML, Al-Qadi MO, Abdulkaki AM, Roberts MB, Eaton CB. Evidence for the vitamin D hypothesis: The NHANES III extended mortality follow-up. *Atherosclerosis*. 2016; 255:96–101 [PubMed: 27855294]
- [20]. Carbone F, Satta N, Burger F, Roth A, Lenglet S, Pagano S, et al. Vitamin D receptor is expressed within human carotid plaques and correlates with pro-inflammatory M1 macrophages. *Vascul Pharmacol*. 2016; 85:57–65. [PubMed: 27555526]
- [21]. Rai V, Agrawal DK. The role of damage- and pathogen-associated molecular patterns in inflammation-mediated vulnerability of atherosclerotic plaques. *Canadian Journal of Physiology and Pharmacology*. 2017;95(10):1245–1253. doi:10.1139/cjpp-2016-0664. [PubMed: 28746820]
- [22]. Su Z, Lu H, Jiang H, et al. IFN- γ -producing Th17 cells bias by HMGB1-T-bet/RUNX3 axis might contribute to progression of coronary artery atherosclerosis. *Atherosclerosis*. 2015;243(2):421–428. doi:10.1016/j.atherosclerosis.2015.09.037. [PubMed: 26520896]
- [23]. Jialal I, Rajamani U, Adams-Huet B, Kaur H. Circulating pathogen associated molecular pattern – Binding proteins and High Mobility Group Box protein 1 in nascent metabolic syndrome: Implications for cellular Toll-like receptor activity. *Atherosclerosis*. 2014;236(1):182–187. doi:10.1016/j.atherosclerosis.2014.06.022. [PubMed: 25063948]
- [24]. Cai J, Yuan H, Wang Q, et al. HMGB1-Driven Inflammation and Intimal Hyperplasia After Arterial Injury Involves Cell-Specific Actions Mediated by TLR4. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2015;35(12):2579–2593. doi:10.1161/atvbaha.115.305789.
- [25]. Kouassi KT, Gunasekar P, Agrawal DK, Jadhav GP. TREM-1; Is It a Pivotal Target for Cardiovascular Diseases? *J Cardiovasc Dev Dis*. 2018;5(3):45. doi:10.3390/jcdd5030045
- [26]. Bangert A, Andrassy M, Müller A-M, et al. Critical role of RAGE and HMGB1 in inflammatory heart disease. *Proceedings of the National Academy of Sciences*. 2015;113(2). doi:10.1073/pnas.1522288113.
- [27]. Kierdorf K, Fritz G. RAGE regulation and signaling in inflammation and beyond. *Journal of Leukocyte Biology*. 2013;94(1):55–68. doi:10.1189/jlb.1012519. [PubMed: 23543766]
- [28]. Takeuchi M Serum Levels of Toxic AGEs (TAGE) May Be a Promising Novel Biomarker for the Onset/Progression of Lifestyle-Related Diseases. *Diagnostics*. 2016;6(2):23. doi:10.3390/diagnostics6020023.
- [29]. Xu L, Wang Y-R, Li P-C, Feng B. Advanced glycation end products increase lipids accumulation in macrophages through upregulation of receptor of advanced glycation end products: increasing uptake, esterification and decreasing efflux of cholesterol. *Lipids in Health and Disease*. 2016;15(1). doi:10.1186/s12944-016-0334-0.
- [30]. Heier M, Margeisdottir HD, Gaarder M, et al. Soluble RAGE and atherosclerosis in youth with type 1 diabetes: a 5-year follow-up study. *Cardiovascular Diabetology*. 2015;14(1). doi:10.1186/s12933-015-0292-2.
- [31]. Nam M-H, Son W-R, Lee YS, Lee K-W. Glycolaldehyde-derived advanced glycation end products (glycol-AGEs)-induced vascular smooth muscle cell dysfunction is regulated by the AGES-receptor (RAGE) axis in endothelium. *Cell Communication & Adhesion*. 2015;22(2-6):67–78. doi:10.1080/15419061.2016.1225196. [PubMed: 27602595]
- [32]. Zhou Z, Wang K, Penn MS, et al. Receptor for AGE (RAGE) Mediates Neointimal Formation in Response to Arterial Injury. *Circulation*. 2003;107(17):2238–2243. doi:10.1161/01.cir.0000063577.32819.23. [PubMed: 12719284]
- [33]. Sakaguchi T, Yan SF, Yan SD, et al. Central role of RAGE-dependent neointimal expansion in arterial restenosis. *J Clin Invest*. 2003;111(7):959–72. [PubMed: 12671045]
- [34]. Reitman JS, Mahley RW, Fry DL. Yucatan miniature swine as a model for diet-induced atherosclerosis. *Atherosclerosis* 1982; 43: 119–132. [PubMed: 7092978]

- [35]. Schwartz RS, Murphy JG, Edwards WD, Camrud AR, Vliestra RE, Holmes DR. Restenosis after balloon angioplasty. A practical proliferative model in porcine coronary arteries. *Circulation* 1990; 82: 2190–2200. [PubMed: 2146991]
- [36]. Inoue K, Kawahara K, Biswas KK, Ando K, Mitsudo K, Nobuyoshi M, Maruyama I. HMGB1 expression by activated vascular smooth muscle cells in advanced human atherosclerosis plaques. *Cardiovasc Pathol.* 2007;16:136–143. doi:10.1016/j.carpath.2006.11.006 [PubMed: 17502242]
- [37]. Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol.* 2001;2:675–680. doi: 10.1038/90609. [PubMed: 11477402]
- [38]. Yang H, Antoine DJ, Andersson U, Tracey KJ. The many faces of HMGB1: molecular structure-functional activity in inflammation, apoptosis, and chemotaxis. *J Leukoc Biol.* 2013; 93:865–873. doi: 10.1189/jlb.1212662. [PubMed: 23446148]
- [39]. Vink A, Schoneveld AH, van der Meer JJ, van Middelaar BJ, Sluijter JP, Smeets MB, Quax PH, Lim SK, Borst C, Pasterkamp G, de Kleijn DP. *In vivo* evidence for a role of toll-like receptor 4 in the development of intimal lesions. *Circulation.* 2002; 106:1985–1990. [PubMed: 12370224]
- [40]. Rao Z, Zhang N, Xu N, et al. 1,25-Dihydroxyvitamin D Inhibits LPS-Induced High-Mobility Group Box 1 (HMGB1) Secretion *via* Targeting the NF-E2-Related Factor 2-Hemeoxygenase-1-HMGB1 Pathway in Macrophages [published correction appears in *Front Immunol.* 2018 Mar 07;9:357]. *Front Immunol.* 2017;8:1308 Published 2017 10 16. doi:10.3389/fimmu.2017.01308 [PubMed: 29085368]
- [41]. Chen S, Law CS, Gardner DG. Vitamin D-dependent suppression of endothelin-induced vascular smooth muscle cell proliferation through inhibition of CDK2 activity. *J Steroid Biochem Mol Biol.* 2010;118(3):135–141. doi:10.1016/j.jsbmb.2009.11.002 [PubMed: 19961935]
- [42]. Li Q, Li J, Wen T, et al. Overexpression of HMGB1 in melanoma predicts patient survival and suppression of HMGB1 induces cell cycle arrest and senescence in association with p21 (Waf1/Cip1) up-regulation via a p53-independent, Sp1-dependent pathway. *Oncotarget.* 2014;5(15):6387–6403. doi:10.18632/oncotarget.2201 [PubMed: 25051367]
- [43]. Zhang H, Yang N, Wang T, Dai B, Shang Y. Vitamin D reduces inflammatory response in asthmatic mice through HMGB1/TLR4/NF- κ B signaling pathway. *Molecular Medicine Reports.* 2017. doi:10.3892/mmr.2017.8216.
- [44]. Higashi T, Sano H, Saishoji T, et al. The receptor for advanced glycation end products mediates the chemotaxis of rabbit smooth muscle cells. *Diabetes.* 1997; 46:463–472. [PubMed: 9032104]
- [45]. Sukino S, Kotani K, Nirengi S, Gugliucci A, Caccavello R, Tsuzaki K, Kawaguchi Y, Takahashi K, Egawa K, Shibata H, Yoshimura M, Kitagawa Y, Sakane N. Dietary Intake of Vitamin D Is Related to Blood Levels of Advanced Glycation End Products during a Weight Loss Program in Obese Women. *J Biomed* 2016; 1:1–4. doi:10.7150/jbm.16497

Fig. 1: Neointimal proliferation following stent insertion is enhanced in vitamin D deficient group and inhibited in Vitamin D supplemented group

OCT imaging was performed in all three experimental group between 16-24 weeks after the initial procedure, with comparable time frame between each group, and demonstrates that vitamin D-deficient swine (Vit. D DEF.) (A) exhibit the greatest levels of in-stent restenosis across the lumen of stented LADs as compared to the vitamin D-sufficient (Vit. D SUF.) (B) and vitamin D-supplemented (Vit. D SUP.) animals (C). H&E results revealing the morphology Vitamin D-deficient swine exhibiting the greatest amount of neointimal hyperplasia (D) as compared to Vitamin D-sufficient (E) and Vitamin D-supplemented animals (F). The images were acquired in 20x magnification. The degree of in-stent restenosis was calculated using OCT integrated software (G) and digital microscopy using a software (H). The number of animals in each group were n=3. Values are presented as mean $\pm$ SEM. Multiple group comparisons were performed by Tukey's multiple comparison test using One-way ANOVA. **Actual p-values noted here were less than 0.05; *p<0.05.**

Fig. 2: Expression of HMGB1 and RAGE upregulated in vitamin D deficient swine

(A-F) Images of the BMS-injured LAD. In vitamin D-deficient swine (Vit. D DEF.) (A), HMGB1+ cells were identified throughout the neointima, clumps of HMGB1+ cells found near lumen and near stent struts compared to the vitamin D-sufficient (Vit. D SUF.) (B) and vitamin D-supplemented (Vit. D SUP.) (C) animals. The greatest number of HMGB1+ cells was found in Vit. D DEF. LAD (G).

Clumps of RAGE+ cells were identified near stent struts of vitamin D-deficient swine (D) and vitamin D-sufficient swine (E) compared to the vitamin D-supplemented swine (F). The greatest number of RAGE+ cells was found in Vit. D DEF. LAD (H). The images were acquired in 40x magnification. The number of animals in each group were n=3. Values are presented as mean ± SEM. Multiple group comparisons were performed by Tukey's multiple comparison test using One-way ANOVA. **Actual p-values noted here were less than 0.01; *p<0.01.**

Fig. 3: Receptor for HMGB1 (TLR4 and TLR2) highly expressed neointima cells in Vitamin D-Deficient swine

(A-F) Images of the BMS-injured LAD. The effect of vitamin D status on TLR4 and TLR2 expression was examined in the LAD post-stent implantation. Immunostaining pattern shows that TLR4 and TLR2 expression is greatest in vitamin D-deficient (Vit. D DEF.) animals (A,D). Vitamin D-sufficient (Vit. D SUF.) animals (B, E) and vitamin D-supplemented animals (Vit. D SUP.) (C, F) demonstrated less stain for TLR4 and TLR2. The greatest number of TLR4+ and TLR2+ cells was found in Vit. D DEF. LAD (G, H). The images were acquired in 40x magnification. The number of animals in each group were n=3. Values are presented as mean ± SEM. Multiple group comparisons were performed by Tukey's multiple comparison test using One-way ANOVA. **Actual p-values noted here were less than 0.01; *p<0.01.**