Lerman et al., Int J Cardiovasc Res 2016, 5:1
http://dx.doi.org/10.4172/2324-8602.1000249

International Journal of Cardiovascular Research

Research Article

A SCITECHNOL JOURNAL

Denosumab could be a Potential Inhibitor of Valvular Interstitial Cells Calcification *in vitro*

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Abstract

Objective: Denosumab is a fully human monoclonal antibody and novel antiresorptive agent that works by binding receptor activator of nuclear factor kappa-β ligand (RANKL) and inhibiting the signaling cascade that causes osteoclast maturation, activity, and survival. We aimed to elucidate the effect of Denosumab in the process of spontaneous and induced calcification in an *in vitro* porcine valvular interstitial cells (VICs) model.

Materials and Methods: VICs were extracted from fresh porcine hearts by serial collagenase digestion. Spontaneous calcification of VICs was increased *in vitro* by adding Na_3PO_4 (3 mM, pH 7.4) and different concentrations (0.1, 1 and 10 ng/ml) of transforming growth factor beta (TGFß). The degree of calcification before and after treatment with Denosumab was estimated by Alizarin Red staining for calcium deposition, and Sirius Red staining for collagen. Colorimetric techniques were used to determine calcium and collagen deposition quantitatively. For statistical analysis we used SPSS and Microsoft Office Excel 2013.

Results: Porcine aortic VICs *in vitro* were induced to calcify by the addition of either 3 mM Na_3PO_4, showing a 5.2 fold increase by 14 days ($P<0.001$), or 3 mM Na_3PO_4 + 10 ng/ml of TGFβ, showing a 7 fold increase by Day 14 ($P<0.001$). Denosumab inhibited induced calcification by 3 mM Na_3PO_4 and 3 mM Na_3PO_4 with the addition of TGFß at either 0.1, 1 or 10 ng/ml to basal levels only at a concentration of 50 µg/ml ($P<0.001$).

Conclusion: This study has proved that Denosumab could be a potential inhibitor of the calcification of VICs *in vitro*. A fuller understanding of the actions of Denosumab may identify a novel therapeutic strategy for clinical intervention against aortic valve calcification and aortic stenosis.

Keywords

Aortic valve calcification disease; Aortic valve interstitial cells; RANK; RANKL; Calcification; Denosumab, Porcine VICs model

Abbreviations: AA: Ascorbic Acid; BGP: Beta-glycerophosphate; BMP: Bone Morphogenetic Protein; CAVD: Calcific Aortic Valve Disease; g: Grams; HBSS: Hank's Balanced Salt Solution; HCl: Hydrochloric Acid; M: Molar; MEM: Minimum Essential Medium (Culture Medium); MIN: Minute/s; ml: Millilitres; MMPs: Matrix Metalloproteinases; mg: Milligrams; mM: Milimolar; ng: Nanograms; OCL: Osteocalcin; OD: Optical Density; OPD: Osteoprotegerin; PBS: Phosphate Buffered Saline; Na_3PO_4: Sodium Phosphate; RANK: Receptor Activator of Nuclear Factor Kappa; RANKL: Receptor Activator of Nuclear Factor K-B Ligand; TIMPs: Tissue Inhibitors of Matrix Metalloproteinases; TGFß: Transforming Growth Factor Beta; µg: Micrograms; µM: Micromolar; VICs: Valvular Interstitial Cells; VSMCs: Vascular Smooth Muscle Cells

Introduction

Calcific aortic valve disease (CAVD) is a slowly progressive disorder with a disease continuum that ranges from mild valve thickening without obstruction of blood flow, termed aortic sclerosis, to severe calcification with impaired leaflet motion, or aortic valve stenosis. CAVD has a multifactorial etiology. On a microscopic level, the aortic leaflets contain disorganized collagen fibers, chronic inflammatory cells, extracellular bone matrix proteins, lipidic proteins and bone mineral [1].

Mechanical and shear stress, endothelial damage and lipid deposition (LDLs) trigger inflammatory events and attract inflammatory cells: monocytes-macrophages and T cells. These cells produce cytokines: transforming growth factor beta (TGFß) which regulates cell proliferation and differentiation; tumor necrosis factor alfa (TNFα) whose primary function is the regulation of the immune cells; and interleukin 2, which is produced by activated T-lymphocytes with growth factor activity [2].

The cyclic movement and mechanical stress of valves require that the tissue has the capacity to repair damage that may occur during normal function. This remodelling is thought to be mediated by the main cell population found in the valve, valvular interstitial cells (VICs), since these cells have reversible and dynamic phenotypes and build the matrix structure in prenatal and postnatal valves [3–5]. VICs play critical functions in maintaining valve homeostasis through secreting extracellular matrix components (e.g., collagen and fibronectin). Valvular interstitial cells (VICs) activated by the inflammatory process are designated myofibroblasts, [1] these cells will develop angiogenic activity and produce matrix remodelling enzymes, such as matrix metalloproteinases (MMPs), proteins that are involved in tissue remodeling and support VICs activation and transformation [6-8]. VICs differentiate into osteoblast-like cells by activation of the receptor activator of nuclear factor kappa -B (RANK) and will favor bone production. During osteoblastic differentiation of VICs an increase of the following bone markers is observed: Alkaline phosphatase (ALP), Osteopontin (OPN), metalloproteinases (MMPs), Runt-related transcription factor 2 (Runx2) and bone morphogenetic protein 2 (BMP-2). All are proteins that indicate that there could be active bone formation [2] (Figure 1). Markers of bone formation are elevated in VICs treated with Receptor activator of nuclear factor-κB ligand (RANKL), a transmembrane, soluble protein expressed by osteoblastic stromal cells, binds to receptor activator of nuclear factor-κB (RANK) and is the primary mediator of osteoclast maturation, differentiation, activation, and survival [7]. RANKL is responsible for osteoclast-mediated bone resorption in a broad range of conditions.

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Received: November 17, 2015 **Accepted:** December 23, 2015 **Published:** January 03, 2016

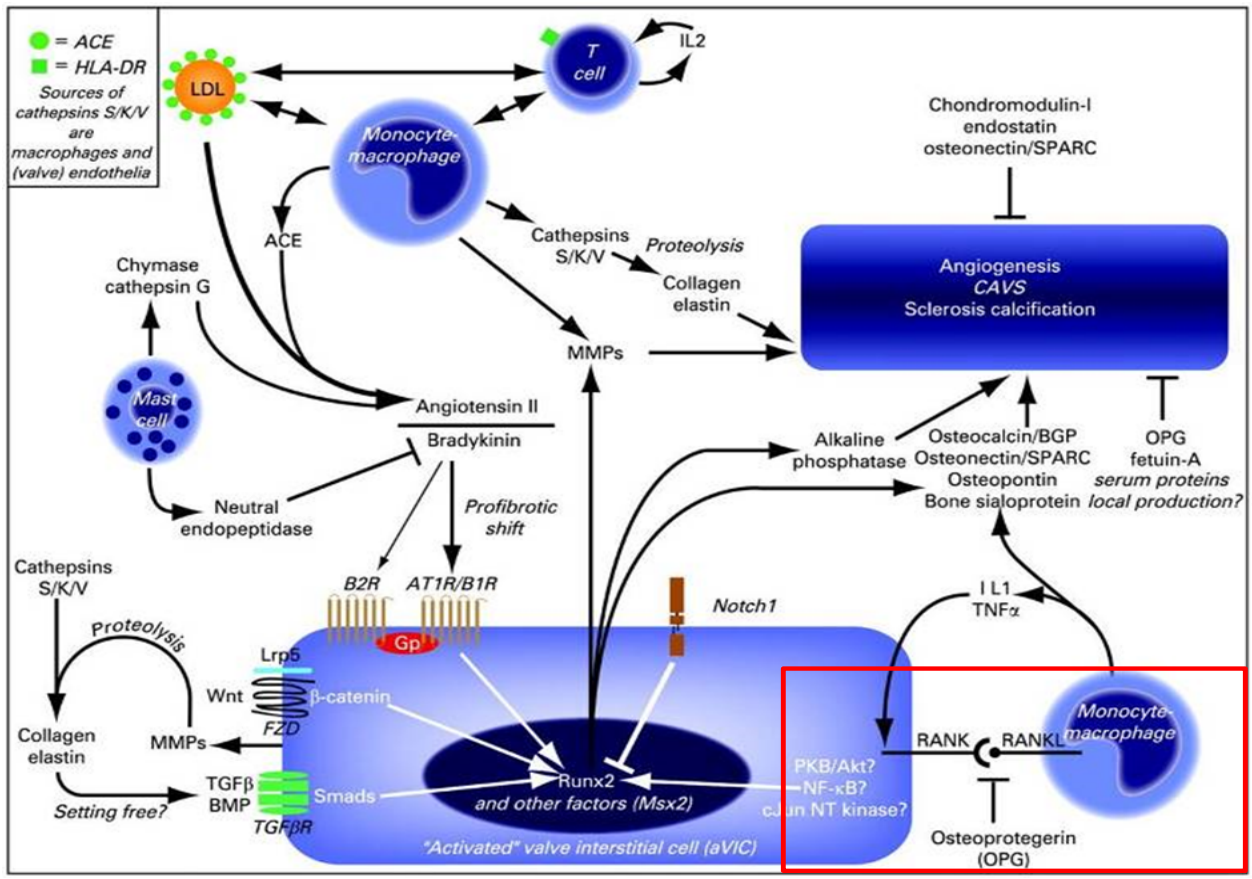


Figure 1: Convergence of regulatory pathways in the activation of VICs. The red rectangle highlights the action of RANKL and the natural inhibition by OPG [2].

Osteoprotegerin (OPG), a soluble signaling RANKL decoy receptor protein that binds RANKL, is the key endogenous regulator of the RANKL–RANK pathway [9,10]. Denosumab (formerly known as AMG 162, Amgen) is a fully human monoclonal antibody (IgG2) designed to bind RANKL with high affinity and specificity and blocks the interaction of RANKL with RANK, mimicking the endogenous effects of osteoprotegerin [11]. Denosumab is used for the treatment of osteoporosis, treatment-induced bone loss, bone metastases, multiple myeloma, and giant cell tumor of bone [12].

Although associated with bone loss and shown to reduce vascular calcification, the effect of Denosumab on calcification of human VICs is unknown. Recently, Denosumab has been shown to reduce calcium deposition in the aorta, but the mechanisms by which it affects ectopic calcification are poorly understood [13]. Furthermore, OPG which is a member of the tumor necrosis factor receptor family, has been shown to stop ectopic calcification *in vitro* by a similar mechanism to Denosumab, but there is still not enough evidence of any effect in reverting the process of calcification. Osteprotegerin mechanism of action is by blocking RANKL-RANK receptor interaction [14,15].

Currently there is no medical treatment approved to treat the progression of CAVD and the only therapy option for severe symptomatic aortic valve stenosis is surgical treatment [16]. However there is still a need of more knowledge of the intrinsic mechanisms involved in valvular calcification to identify future preventive and therapeutic targets.

Aim and Hypothesis

Normal gene expression is altered in diseased aortic valve tissues. Recognized pathways in bone mineralization are known to be involved in the formation of calcific aortic stenosis but we hypothesized that a novel pathway specific to vascular cell mineralization would also exist. Osteoprotegerin shown to stop ectopic calcification *in vitro* by a similar mechanism to Denosumab but there is still not enough evidence of any effect in reverting the process of calcification. Denosumab is designed to target the receptor activator of nuclear factor-κB ligand (RANKL), and it is used in the treatment against osteoporosis. RANKL is known to regulate vascular calcification [17]. Valvular calcification is recognized as a fundamental component of calcific aortic stenosis and further insight into the mechanism by which it occurs is required to develop novel strategies to combat it. We aimed to elucidate the effect of Denosumab in the process of spontaneous and induced calcification in an *in vitro* porcine valvular interstitial cells (VICs) model.

Material and Methods

Obtaining aortic valve leaflets

Ten healthy, 6 month old fresh porcine (*Sus scrofa domestica*) hearts were collected from local abattoir (Grampian Country Pork Halls Ltd, West Lothian, EH52 5AW) within 3 hours of sacrifice. The study was approved by the Lothian Research Ethics Committee (REC reference: 08/S1101/23) and conducted under the UK Home Office guidelines for the care and use of laboratory animals.

Under sterile conditions aortic valve leaflets of the animal hearts were dissected 2 mm away from the aortic annulus under microscopic inspection. The remaining tissue was discarded. The obtained leaflets were rinsed in sterile cold saline to remove blood. The leaflets were placed in a Petri dish containing sterile Hanks' balanced salt solution (HBSS, Gibco® cell culture). The principal function of a balanced salt solution (HBSS) is to keep the pH and the osmotic balance, as well as to provide the porcine cells with water and essential inorganic ions.

Cell isolation and cultures

Primary valvular interstitial cells (VICs) were extracted from the prepared leaflets by enzyme digestion with 5 ml of 0.6 mg/ml collagenase type 2. Gentle shaking was maintained throughout the first step for 20 minutes at the temperature of 37°C. Digested tissue was mixed with universal vortex for 30 seconds, after which it underwent a second digestion with 5 ml of 0.6 mg/ml collagenase type 2 digestion in HBSS medium for additional 40 minutes at the same temperature. The quantity of clostripan (a proteinase that cleaves the proteins at the level in which the amino acid arginin is bonded to the carboxilpeptide) is higher in the enzyme collagenase type 2 than in other collagenases. This quality optimizes its activity for cell isolation of aortic valves [18].

After neutralisation of the collagenase type 2, the cells were cultured in growth medium consisting of Dulbecco's modified Eagle medium (α MEM, Sigma, Dorset, UK) supplemented with 10% heat inactivated fetal bovine serum (FBS; Gibco. Invitrogen, Milan Italy). The media was protected against contamination with 1% Gentamicyn antibiotic.

Cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C in T 75 tissue culture flasks (Greiner Bio-One GmbH, Germany) until confluent. All reagents and biologic cultures were prepared under sterile conditions in a fume hood. A hemocytometer was used to assess cell counts before plating wells at a density of $1.5x10^5$ cells x well and incubated 3-4 days until confluent. Passage 2 cells were then treated with promoters and inhibitors of calcification and samples taken at day 0, 7 and 14 for molecular studies. Wells at a density of $1.5x10^5$ cells x well of untreated porcine VICs were used as a control. Medium was changed every 3-4 days during the incubation period. Experiments included a time-course study under different promoters and inhibitors of calcification over a 14 day period.

Calcification promotion and inhibition in VICs

Induced calcification in porcine VICs was carried out with sodium phosphate and TGFß1 (R&D Systems, Minneapolis, MN). 3 mM Sodium Phosphate (Na_3PO_4; pH 7.4) and 0.1 ng/ml to 10 ng/ml of TGFß were tested as inducers of calcification and the effect of Denosumab as an inhibitor of calcification was analyzed *in vitro*. The effect of Ascorbic acid, beta-glycerophosphate and dexamethasone were also analyzed as a potential promoter of calcification. Six well plates were seeded at a density of 1.5×10^5 cells per well and incubated 3-4 days until confluent. Cells were then treated with 3 mM Na_3PO_4 ± Denosumab (0.5 µg/ml, 5 µg/ml and 50 µg/ml) and samples taken at day 0, 7 and 14 for molecular studies. Concentration of Etidronate (a bisphosphonate), which can reduce osteoclastic activity *in vivo* in humans, was considered a potential inhibitor of VICs calcification, and was tested as follows: 1-100 µg/ml, diluted in distilled water.

Detection of calcification (Alizarin Red)

Calcium deposition was detected by staining the cells, matrix monolayer with the dye Alizarin Red (1.2 dihydroxyanthraquinone), which is an organic compound used in biochemical assays to determine calcium deposition quantitatively by colorimetric techniques [19]. Cells, isolated from dissection of porcine aortic valve leaflets, were washed twice with phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 5 minutes at 4°C and then stained with 2% Alizarin Red at a pH of 4.2 for a period of 5 min at room temperature, following a rinse with distilled water. It is possible in this way to extract the dye from the stained monolayer and then assay the stained structures. The remaining calcium bound Alizarin Red was extracted by adding 10% cetylpyridium chloride for 10 min. The determination of the optical density (OD) was carried out at 570 nm by measuring the absorbance by using a spectrophotometer (Multiskan Ascent, Thermo Electron Corporation,Vantaa, Finland).

The stained matrix was decalcified at a pH<7 with a solution of Hydrochloric acid (HCl) 0.6 N for a 24 hours period. The free calcium found in the solution was measured by using a colorimetric technique.

The commercial kit (Randox Laboratories Ltd, Crumlin, and County Antrim, UK) was corrected for total protein concentration. The total protein quantity of cell cultures was determined by using the BioRad protein assay reagent (Hertfordshire Laboratories UK) and the entire assay was carried out following the Bradsford based dye binding technique. Gamma globulin was used as a standard protein for the experiment [20].

A calcium sample with a concentration 2.5 M was used as a positive control. A negative control is made of a 0.6 N concentration of HCl. All the samples were set up in duplicate. The OD reading was at 570 nm and data was normalized by the protein content detected in the cellular layer (protein assay analysis BioRad).

Calculation of calcium concentration

Calcium concentration = (read absorbance of the sample/read absorbance of the standard) x standard concentration (2.5 mmol/l)

Calculation of collagen concentration (Sirius Red)

Sirius Red is a metal complex cationic and acidic dye. The molecular structure makes it extremely hydrophilic and this property enables it to make a strong complex combination and stain collagen fibers [21].

Cells were first fixed in 1% Paraformaldehyde and then they were stored in picric acid phosphate buffered saline (PBS) at a pH of 7.6. Afterwards, cells underwent a wash in saline water and they were left to dry. Subsequently, cells were stained with picric red dye for one hour (Sirius Red 0.5 grams) in picric acid (500 ml)). Finally, cells were washed extensively with running water and hydrochloric acid 0.001 M was added to remove the unbound dye, and then they were digested with sodium hydroxyl 0.1 M (500 µl per well) for 30 minutes.

Assessment of toxicity

Cell growth was measured using Roche xCELLigence technology. Differences in VICs growth rate were observed following treatment with Na_3PO_4 3 mM and Denosumab at a concentration of 5 µg/ml and 50 µg/ml over a culture period of 6 days. Degrees of toxicity were assessed statistically.

Statistical analysis and software

All data was analyzed initially by ANOVA (general linear model analysis). After ANOVA, non-parametric tests were performed. Linear variables were compared using Student's t-test and the following

statistics methods were applied as required: Levene's Test for Equality of Variances, Scheffe for multiple comparisons and post Hoc Dunnett to compare each group with the control group. All results are expressed as the mean ± standard deviation of the mean (SD) which is displayed as Y error dot plots on graphics with a confidence interval of 95%. Software Programmes: IBM SPSS and Microsoft Office Excel 2013 for statistical analysis. A P value of<0.05 was considered statistically significant. The Multiskan Ascent Machine (Thermo Electron Corporation, Thermo Fisher Scientific, Massachusetts, USA) was used to perform colorimetry and the Ascent software version 2.6 was used to perform, store and analyse colorimetric data.

Results

Examination of cell viability under microscope

Porcine VICs spontaneously calcify *in vitro*, and after a period of seven days it is possible to observe microscopic changes which are related to the presence of calcification nodules (Figure 2A). The peak of calcification, confirmed later by calcium bound Alizarin Red in cultured cells, appears after 14 days with an increase in the number and size of microscopic nodules (Figure 2B). These spontaneous calcification changes in porcine VICs show that they are a useful model for studying the calcification process of porcine aortic valve leaflets in detail. VICs cultured in a non-calcified medium proliferated steadily throughout the 14 day study period.

Quantification of calcium and collagen

Peak of spontaneous calcification changes was observed by day 14 and this was confirmed by quantification of calcium deposition detected by Alizarin Red staining and collagen deposition detected by Sirius Red staining. From day 0 to day 14, there was a 3.76-fold increase in calcium deposition (day 0: 0.202 ± 0.050, day 14: 0.761 ± 0.060 OD; p<0.001) and a 35.5-fold increase in collagen deposition (day 0: 0.044 ± 0.004, day 14: 1.530 ± 0.046 OD; p<0.001) (Figure 3).

Effect of Ascorbic Acid, Beta-glycerophosphate and Dexamethasone as calcification promoters

Experiment in porcine VICs showed that growth medium supplemented with the Ascorbic acid (AA) at a concentration of 50 µg/ml and beta-glycerophosphate (BGP) at a concentration of 10 mmol/l over a 14 days culture affected the health of the cells significantly, with no increase in the number of nodules, while dexamethasone (a potent synthetic glucocorticoid) at a concentration of 100 nmol/l killed the cells quickly. Dexamethasone is known to be a strong promoter of osteoblastic differentiation *in vitro*. However, the mechanism of action is still unclear [22]. Additionally, it was observed under microscope that only controls and cells treated with sodium phosphate (Na_3PO_4), and not those treated with either AA or BGP, formed spots of calcification from day 7.

Effect of Etidronate in spontaneous calcification of porcine VICs

Etidronate is a bisphosphonate which inhibits calcification of vascular smooth muscle cells (VSMCs) [23]. No obvious significant changes in calcium deposition by Alizarin Red staining were observed for Etidronate treated cell cultures of porcine VICs at a concentration of 1 and 10 and µg/ml (Figure 4) but at a maximum concentration of 100 µg/ml it was observed that the vitality of the cells was slightly affected with a reduction in the total number of cells counted under the hemocytometer.

Effect of Sodium phosphate, TGFß and Denosumab in porcine VICs

OD at 570 nm of the control culture of VICs with normal medium by day 14 was 0.41 ± 0.01. Addition of either 3 mM Na_3PO_4 or 3 mM Na_3PO_4 + 10 ng/ml TGFβ induced a 5.2 fold (OD=2.1 ± 0.21, p<0.001) and a 7 fold (OD=2.87 ± 0.27, p<0.001) increase of calcification by 14 days respectively (Figure 5). With the addition of 50 µg/ml of Denosumab to those cultures we could achieve inhibition of induced calcification by Na_3PO_4 (OD=0.28 ± 0.01, P<0.001) and Na_3PO_4 + TGFß (OD=0.29 ± 0.01, P<0.001) to baseline levels (Figure 5). Denosumab inhibits induced calcification by 3 mM Na_3PO_4 and 3 mM Na_3PO_4 with the addition of TGFß at either 0.1 ng/ml, 1 ng/ml or 10 ng/ml to basal levels only at a concentration of 50 µg/ml (P<0.001) (Figures 6 and 7).

Studying the toxicity of Denosumab

It was analysed the normalized cell index of cultured porcine VICs to assess cell growth under treatment with different concentration of drugs. No significant differences in cultured VICs growth rate were observed following treatment with Denosumab at a concentration of 5 µg/ml, 50 µg/ml or 3 mM Na_3PO_4 (Figure 8) over a 6-day time period, thus indicating no considerably toxicity. Untreated porcine VICs were used as a genuine control which showed no growth rate differences over the same time period. Cell growth rate was investigated using Roche xCELLigence technology.

Discussion

This investigation into novel regulators and inhibitors of aortic

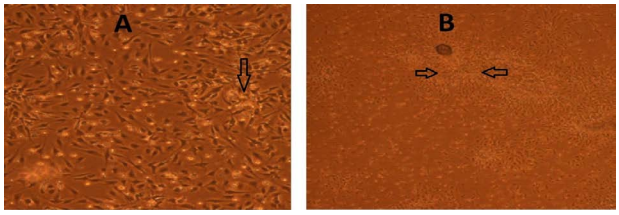


Figure 2: Porcine VICs after 7, 14 days' culture. 2A: Showing images of nodule morphology. 2B: Showing an increase in the number of nodule formations (arrows).

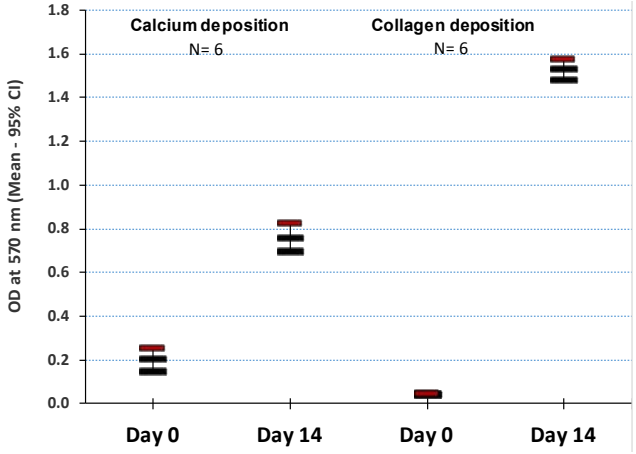
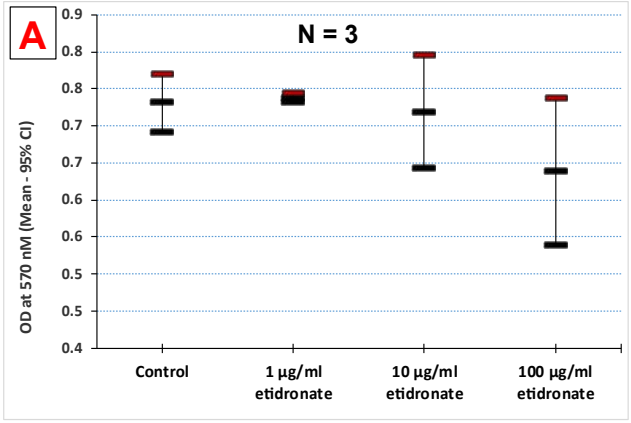


Figure 3: Spontaneous calcification of porcine VICs. Showing quantification of calcium deposition by Alizarin Red and collagen deposition by Sirius Red staining.



B

Multiple Comparisons

Dependent Variable:

(I) Group			Mean Difference (I-J)	Std. Error	P	95% Confidence Interval	
						Lower Bound	Upper Bound
Dunnett t (2-sided)[b]	1 µg/ml etidronate	Control	,006317	,021499	,982	-,05559	,06823
	10 µg/ml etidronate	Control	-,011783	,021499	,903	-,07369	,05013
	100 µg/ml etidronate	Control	-,092650*	,021499	,007	-,15456	-,03074

*. The mean difference is significant at the 0.05 level.

b. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Figure 4: Spontaneous calcification of VICs. Showing: A: Quantification of calcium deposition of porcine VICs under Etidronate: 1, 10 and 100 µg/ml. B: Multiple comparisons analysis.

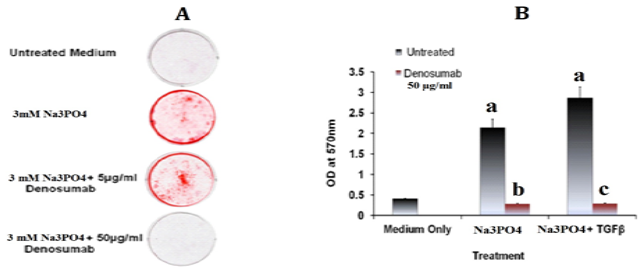


Figure 5: Denosumab inhibits calcification of VICs. Showing (A) Alizarin Red staining for calcium deposition, and (B) quantification of calcium deposition: a denotes P < 0.001 when compared to the medium only control; b and c denote P < 0.001 when compared to cells not treated with Denosumab.

valve calcification was undertaken with a view to establish a starting point for the development of potential medical treatment for calcific aortic stenosis. This system mimics several mechanisms that take place *in vivo*. VICs are the main population of cells found in cardiac valves. These resident fibroblastic cells play important roles in maintaining proper valve function, and their dysregulation has been linked to disease progression in humans. Despite the critical functions of VICs, their cellular composition is still not well defined for humans and other mammals. Given the limited availability of healthy human valves and the similarity in valve structure and function between humans and pigs, porcine VICs can be a good model to study the process of CAVD. Our initial results revealed that porcine VICs calcify spontaneously with demonstrable calcium deposition by day 14. Furthermore, Collagen deposition by day 14 showed an enormous growth. Advancement of calcification is associated with rising Collagen levels and will set up the matrix in which VICs transdifferentiation process takes place. TGFß is known to show osteogenic and apoptotic activity. Cells which secrete TGFß can usually at the same time have receptors for this protein and thus autoregulate its secretion. This ability is known as autocrin secretion. TGFß at different concentrations and 3 mM Na_3PO_4 were used to increase calcification of selected cell cultures of porcine VICs.

Denosumab, a human monoclonal antibody, is designed to target the receptor activator of nuclear factor kappa–B ligand (RANKL), which is attached to the membrane of the osteoblasts and osteoclasts. Denosumab is used in the treatment against osteoporosis. Additionally, thanks to its mechanism which blocks the receptor RANKL, it will neutralize the activation of RANK receptor attached to the membrane of pre-osteoclast cells. In relation to this, more research is needed to address the interaction between RANK receptor and Denosumab in porcine VICs. In our recent study we demonstrated that Denosumab showed to inhibit induced calcium deposition to basal levels in porcine VICs culture. Would this be achieved by inhibiting VICs activation through blocking RANK-RANKL interaction?

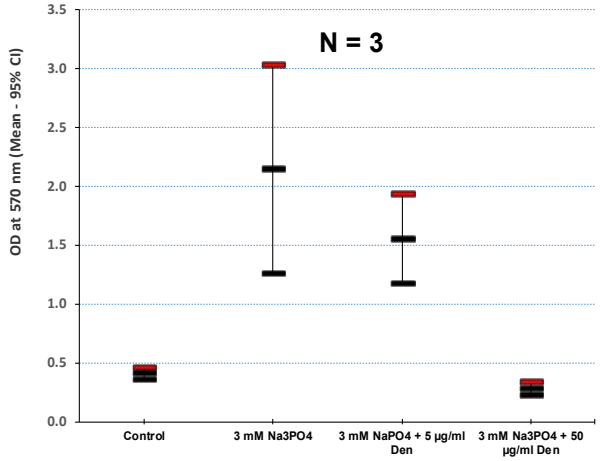


Figure 6: The effects of Denosumab on calcification of VICs. Showing levels of calcium build up determined by Alizarin Red staining of VICs treated with alpha MEM based calcifying medium with 5ug/ml or 50ug/ml of Denosumab. (Den: Denosumab).

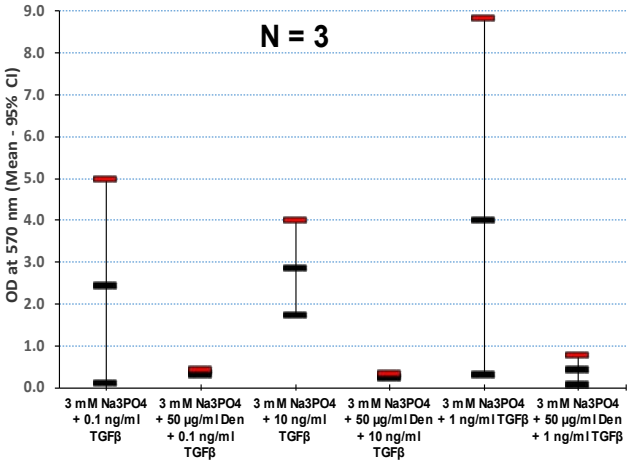


Figure 7: Denosumab and TGFβ experiment in pig (VICs). Denosumab (50 µg/ml) inhibits induced calcification by Na_3PO_4 and TGFß (0.1 ng/ml; 1 ng/ml and 10 ng/ml) to basal levels.

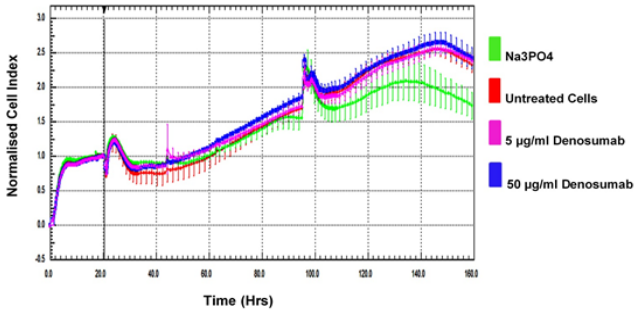


Figure 8: Showing cell growth rate of porcine VICs culture following treatment with Denosumab at 5 and 50 µg/ml, 3 mM Na_3PO_4 and untreated control.

Etidronate is a biphosphonate inhibitor of osteoclastic activity in humans and currently an elective treatment against osteoporosis. Our research showed that it does not affect calcification in porcine VICs up to a concentration of 10 µg/ml, but at a maximum concentration of 100 µg/ml, it was observed that cells' vitality was slightly affected with a limited reduction in calcium deposition (Figure 4).

Porcine VICs have shown good response to induced calcification with Na_3PO_4, showing a 5.2 fold increase by day 14 ($P<0.001$) in calcium deposition (Figure 3). This correlates well with an increase in VICs activity and transdifferentiation of its phenotype that was shown in our previous research.

Denosumab inhibits induced calcification of porcine VICs by Na_3PO_4 and TGFβ at a concentration of 50 µg/ml, but not at a lower concentration (Figures 5-7). This is reflected by the Alizarin Red staining studies which show a significant decline in the calcium levels of VICs. Denosumab has no toxic effect on VICs at a concentration of 50 µg/ml over a 6-day time period (Figure 8).

In conclusion, a better understanding of the mechanism of action of Denosumab may identify a novel therapeutic strategy for medical treatment against CAVD and will allow physicians to tackle the progression of aortic stenosis by other means than those of surgical treatment.

Summary

It is possible to conclude that porcine VICs are a good model to study the process of CAVD. Calcification of VICs can be increased up to a maximum level by the addition of Na_3PO_4 3 mM and TGFβ 10 ng/ml + Na_3PO_4 3 mM. Denosumab inhibited induced calcification to baseline with no toxic effect on cells. A fuller understanding of the mechanisms of action of Denosumab may identify a novel therapeutic approach for clinical treatment and it would supplement the current surgical approach. It should be noted that extrapolation of the results obtained in an *in vitro* porcine model to humans should be done very carefully, as species variations are likely to exist.

Acknowledgment

We wish to thank all the support from Dr Neil Mackenzie that unfortunately passed away after falling 2,000ft down a mountain while ice climbing in Canada (RIP). We are very grateful to Dr. Pablo Andrés Salgado, Consultant in Statistic at Pan American Health Organization (PAHO)/WHO for the statistical analysis. We would also like to thank Dr Kiddy L. Ume for helping with the edition of the manuscript.

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