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Research Article

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Using Na_3PO_4 to Enhance *In vitro* Animal Models of Aortic Valve Calcification

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Abstract

Background/Objectives: The pathogenesis of calcific aortic valvular disease (CAVD) involves an active inflammatory process of valvular interstitial cells (VICs) characterized by the activation of specific osteogenic signaling pathways and apoptosis. This process can be studied by analyzing certain molecular markers and gene expression pathways of spontaneous calcification. The purpose of our study is to investigate the role of sodium phosphate (Na3PO4) as a calcification promoter, with the aim of improving in vitro animal models for testing potential calcification inhibitors.

Materials and Methods: VICs were extracted from 6 healthy 6-month-old fresh porcine hearts by serial collagenase digestion. Quantitative polymerase chain reaction (qPCR) was used to quantify trans-differentiation of genes of interest during spontaneous calcification of VICs. Spontaneous calcification of VICs was increased by adding Na_3PO_4 (3 mM, pH 7.4). The degree of calcification 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. Additionally, the enzymatic activity of alkaline phosphatase (ALP) was measured by a kinetic assay. For statistical analysis we used SPSS and Microsoft Office Excel 2013.

Results: Porcine VICs calcify spontaneously with demonstrable calcium and collagen deposition. In this study we observed an increase of calcium and collagen deposition from day 0 to day 14 (calcium: 376%; P<0.001, collagen: 3553%; P<0.001). qPCR analysis of mRNA by day 14 showed the following results: α-actin, a marker of myoblast phenotype, was increased to 1.6-fold; P<0.001. Runx2, an osteoblast marker, rose to 1.3 fold; P<0.05, TGF-β, a promoter of osteogenesis, increased to 3.2-fold; P<0.001, and RhoA, a regulator of nodular formation in myoblasts, increased to 4.5-fold; P<0.001, compared to their levels at day 0. RANKL mRNA and calponin did not change significantly. Treatment of porcine VICs with Na3PO4 (3 mM, pH 7.4) led to a marked increase in calcium deposition by day 14 (522%; P<0.001), and a significant increase in ALP activity by day 7 (228%; P<0.05). There were no significant changes in ALP activity between the groups by day 14.

Conclusion: This study has demonstrated the upregulation of some specific molecules during spontaneous calcification of aortic VICs with an active increase of calcium, collagen and ALP activity. In this in vitro model it was possible to increase spontaneous VICs calcification with Na_3PO_4 (3 mM, pH 7.4) to a level in which inhibitors of calcification could be tested to identify a novel potential therapeutic strategy against calcific aortic stenosis.

Keywords:

Aortic valve calcification; Aortic valve interstitial cells; Markers of calcification; Markers of trans-differentiation; Gene regulation; Aortic porcine model; Sodium phosphate

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Abbreviations: ALP: Alkaline Phosphatase; CAVD: Calcific Aortic Valve Disease; cDNA: Complementary DNA; DMEM: Dulbecco's Modified Eagle Medium (Culture Medium); DNA: Deoxyribonucleic Acid; FBS: Foetal Bovine Serum; HBSS: Hank's Balanced Salt Solution; M: Molar; MEM: Minimum Essential Medium (Culture Medium); MIN: Minute/S; Ml: Millilitres; MMPs: Matrix Metalloproteinases; Mg: Milligrams; mM: Milimolar; mRNA: Messenger Ribonucleic Acid; ng: Nanograms; OD: Optical Density; OPD: Osteoprotegerin; pNPP: Para Nitrophenyl Phosphate; PBS: Phosphate Buffered Saline; PCR: Polymerase Chain Reaction; PFA: Paraformaldehyde; qPCR: Quantitative Polymerase Chain Reaction; Na_3PO_4: Sodium Phosphate; RANK: Receptor Activator of Nuclear Factor Kappa; RANKL: Receptor Activator Of Nuclear Factor K-B Ligand; RhoA: Ras Homolog Gene Family, Member A; RNA: Ribonucleic Acid; RT-PCR: Reverse Transcription-Polymerase Chain Reaction; Runx2: Runt-Related Transcription Factor 2; TGF-ß: Transforming Growth Factor Beta; TNFα: Tumor Necrosis Factor Alpha; µg: Micrograms; µM: Micromolar; VICs: Valvular Interstitial Cells

Background

Accumulation of fibrotic tissue and calcium in the aortic valve leads to decreased pliability and narrowing of the valve orifice. Over the past several decades, the etiology of aortic valve stenosis has changed considerably. The decline of rheumatic heart disease and increased longevity in industrialized countries has resulted in a pattern shift from rheumatic aortic stenosis to degenerative calcification as a dominant etiology of aortic valve stenosis. Severe stenosis of calcific aortic valve disease (CAVD) is the most common cause of valve replacement surgery in Europe and North America affecting > 2% of individuals over age 65 and > 50% of those over age 85 years [1,2]. Recent evidence suggests that CAVD is due to an active inflammatory process affecting the valve and leading to the activation of the receptor activator of nuclear factor kappa-B (RANK) in valvular interstitial cells (VICs) [1], leading to osteoblastic transformation with bone formation.

Calcific aortic stenosis is more common in patients with osteoporosis [3,4]. Inflammatory processes and hyperlipidemia have been shown to have significant roles in the development of osteoporosis, CAVD and vascular disease [5-7]. Interestingly, the expression of RANKL by osteoblast cells is actively involved in the activation and differentiation of osteoclast cells [5]. RANKL levels normally rise with age and can predict cardiovascular events in humans, while the physiologic inhibitor of RANK, Osteoprotegerin (OPG) deficit can lead to vascular calcification in animal models [8,9]. In this present study in vitro study we aim to characterize some of the specific markers and gene expression pathways of spontaneous calcification in porcine VICs. Additionally, we investigate the role of Sodium phosphate (Na_3PO_4) as a calcification promoter to improve this model for testing potential calcification inhibitors.

Material and Methods

Aortic valves

Six healthy, 6-month-old fresh porcine (*Sus scrofa domestica*) hearts were collected from local abattoir (Grampian Country Pork

Halls Ltd, West Lothian, EH52 5AW). Aortic valve leaflets of the animals were dissected 2 mm away from the aortic annulus under microscopic observation and sterile conditions. The obtained leaflets were rinsed in sterile cold saline to remove blood. Endothelial cells were scraped off using a scalpel blade. The leaflets were denuded of the endothelial layers and were placed in a Petri dish containing sterile Hanks' balanced salt solution (HBSS, Gibco® cell culture). 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.

Cell 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.

After neutralization 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.

Six well plates were seeded at a density of $1.5x10^5$ cells per well and incubated 4 days until confluent. Morphology of cultured VICs was observed under microscope and cell count was assessed by a haemocytometer.

Three well plates were then treated with Na_3PO_4 (3 mM, pH 7.4) to increase calcification. The rest of the well plates were used as control. Cells were cultured for 14 days in a humidified atmosphere of 95% air and 5% CO_2 at 37°C in T75 tissue culture flasks (Greiner Bio-One GmbH, Germany) until confluent. The medium was renewed every 3 days during the period in which the incubation took place. Samples were taken at day 0, 7 and 14 for molecular studies. All reagents and biologic cultures were prepared under sterile conditions in a fume hood.

Molecular studies in porcine valve interstitial cells (VICs)

The standard curve for β-Actin in porcine VICs was linear (on a logarithmic scale). The curve showed that this primer works well with porcine samples (Figure 1). As a consequence, this housekeeping gene could be used as a reference for other genes assayed using polymerase chain reaction (PCR).

Purified mRNA from VICs was extracted, and reversely transcribed into complementary DNA (cDNA) at day 0, 7, 14 for molecular studies in a Thermo Hybrid Gradient PCR Block machine (Thermo Scientific, Loughborough, UK) (Table 1).

All quantitative PCRs (qPCR) were performed using the Stratagene Mx 3000P, real time multiplexes qPCR system (Stratagene, CA, USA).

Detection of calcification

Calcium deposition was detected by staining the cells and the matrix monolayer with Alizarin Red dye (1.2 dihydroxyanthraquinone), which is an organic compound used in biochemical assays to determine calcium deposition quantitatively by colorimetric techniques [10]. The isolated cells were washed twice with phosphate buffered saline (PBS) and 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. The remaining calcium bound Alizarin Red was extracted by adding 10% acetylpyridium chloride for 10 min. The determination of the optical density (OD) was carried out by measuring the absorbance at 570 nm using a spectrophotometer (Multiskan Ascent, Thermo Electron Corporation,Vantaa, Finland).

Calculation of collagen concentration

Cells were first fixed in 1% paraformaldehyde (PFA) and then they were stored in picric acid phosphate buffered saline at a pH of 7.6. Cells underwent a wash in PBS and after drying, they were stained with 50 mg Sirius Red dye in 500 ml picric acid for one hour. Finally, cells were washed extensively with running water and hydrochloric acid 0.001 M was added to remove the unbound dye, which was followed by a sodium hydroxyl 0.1 M (500 µl per well) digestion for 30 minutes.

Protein content

The total protein concentration was obtained by comparing the absorbance of each sample to the standard curve, which was calculated with known concentrations of serum albumin (BSA) 2 mg/ml (Biorad). The standards and the samples were diluted in lyses buffer. The quantification of proteins was assessed by micro-protein assay (Biorad, Hertfordshire, UK).

Alkaline Phosphatase (ALP) activity

Cells membrane was disrupted by lyses with 0.9% sodium chloride and 0.2% Triton X -100 and centrifuged at 12000 rpm for 15 min at 4°C. The supernatant was analyzed for protein content. The samples were defrosted and analyzed by using the ALP Kit (Thermo trace, Melbourne, Australia). All the samples were analyzed in duplicate. Distilled water was used as a blank sample. A 405 nm filter was required for colorimetric analysis. A standard curve, obtained from previous data, was used to work out the final results. The following formula was applied for ALP activity: ALP activity (nm) = 40.5423* OD – 0.21133 (OD is the optical density reading at 450 nm). The enzymatic activity was determined by measuring the splitting of a complex molecule into simpler molecules at 405 nm with 10 mM p-nitrophenyl phosphate (pNPP).

The concentration of protein was calculated by multiplying the dilution factor used in preparing the samples. The determination of the total amount of protein in mg was obtained by multiplying the concentration of protein by the volume in the original sample (0.3 ml). To avoid discrepancy resulting from more cells or protein content in a sample, ALP was normalized against total protein (dividing ALP/Prot). The final ALP activity was expressed in in the following units: nM pNPP hydrol/min/mg protein.

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

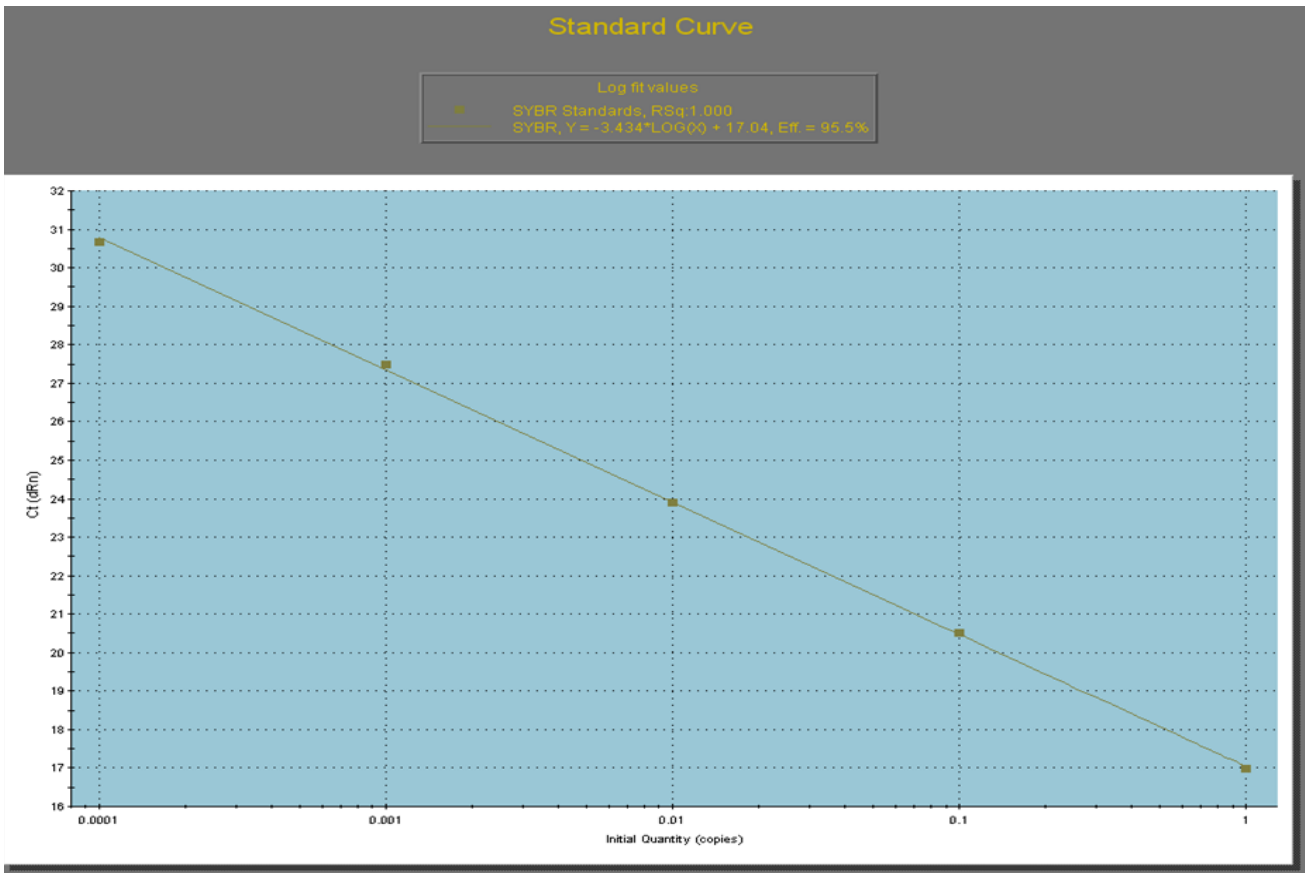

Figure 1: The standard curve for β-Actin

Table 1: Synthesis Report for forward and revers porcine primers.

Target	Forward	Reverse
ß-Actin	5-ATG GTG GGT ATG GGT CAG AA-3	5-CGG AGC TCG TTG TAG AAG GT-3
α-Actin	5-AACAACCACAGAACCACAAG-3	5-TGACCTGCGCATTAACC-3
RhoA	5-ACCAGTTCCCAGAGGTGTATGT -3	5-TTGGGACAGAAATGCTTGACTTC -3
Run x2	5ß-TACAAACCATACCCAGTCCCTGTTT-3	5ß-AGTGCTCTAACCACAGTCCATGCA-3
TGF-ß	5-CGAGCCAGAGGCGGACTAC- 3	5-TTGGTTGCCGCTTTCCA- 3
Calponin	5-GGCTGAGGTCAAGAACAAGC-3	5-CCAGT TCTGGGTGGACTCAT-3
RANKL	5-GAAAAAGCCATGGTGGAAGGT-3	5-GGGATGTCTGTGGCGTTGAT-3

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. MxPro 3000P was used to analyze and store data from qPCRs. A P value of < 0.05 was considered statistically significant.

Results

Peak of spontaneous calcification and collagen deposition were assessed by Alizarin Red and Sirius Red staining, respectively, and quantified using a colorimetric technique at day 0 and day 14. 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) (Figures 2 and 3). Treatment of VICs with Na_3PO_4 (3 mM, pH 7.4) resulted in the development of calcium nodules observed microscopically, and a 5.2-fold increase in calcification by day 14 (control group: 0.41 ± 0.02, treated group: 2.15 ± 0.36 OD; p<0.001) (Figure 4).

ALP activity by day 7 was significantly higher in the Na_3PO_4 treated group indicating an increased calcification in VICs (untreated group ALP activity: 1.48 ± 0.35 and Na_3PO_4 treated group ALP activity: 3.37 ± 1.28 nM pNPP Hydrol/min/mg Protein; p< 0.05). While ALP activity continued to increase in the untreated group through day 8-14, (2.15 ± 1.10 nM pNPP Hydrol/min/mg Protein), a significant decline was being observed in the treated group (1.13 ± 0.08 nM pNPP Hydrol/min/mg Protein; p<0.05). As a result, there was no significant difference between the ALP activity of the two groups by day 14 (p=0.073) (Figure 5).

Gene expression studies in porcine VICs

mRNA levels were analyzed by qPCR during spontaneous calcification. There were significant changes in the expression

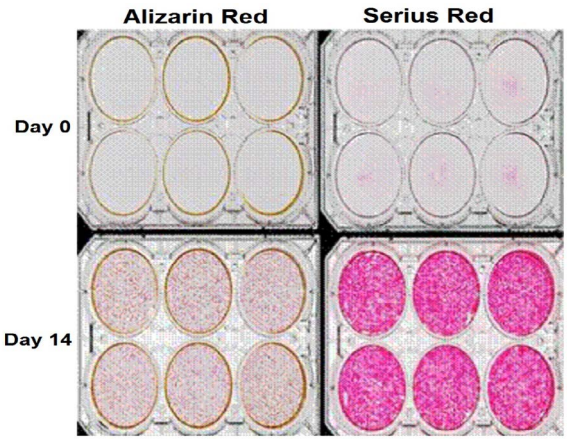

Figure 2: Spontaneous calcification of porcine VICs. Showing Alizarin Red and Sirius Red staining for calcium and collagen deposition of cell monolayers by day 14.

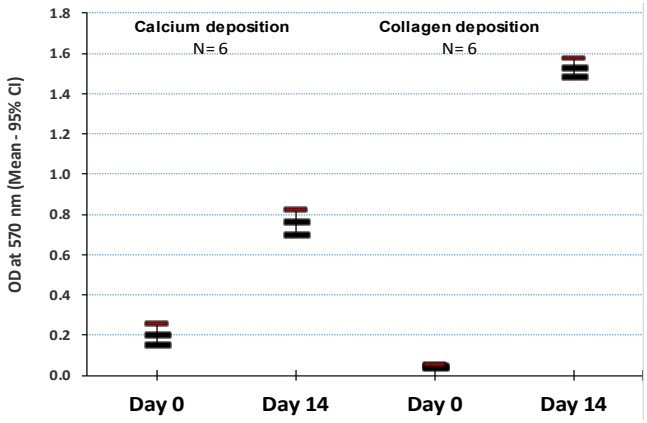

Figure 3: Spontaneous calcification of porcine VICs. Showing quantification of calcium deposition by Alizarin Red and collagen deposition by Sirius Red staining.

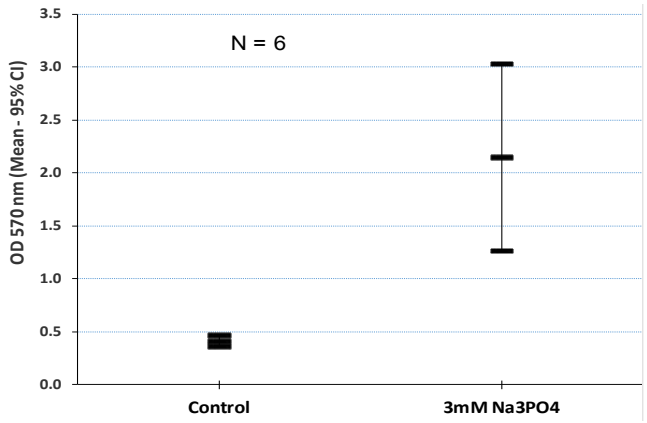

Figure 4: The effect of Na_3PO_4 treatment on calcium deposition. A significant increase in calcium deposition was observed in the treated group comparing to the control group by day 14.

of certain markers of VICs cell trans-differentiation. During spontaneous calcification, VICs expression of α-actin, which is a marker of myofibroblast phenotype, showed a 161% increase from day 0 to 14. (relative change in expression day 0: 1 ± 0.27, day 14: 1.61 ± 0.43; p<0.001). Meanwhile, expression of RhoA, which is a regulator of nodule formation in myofibroblasts increased by 449% (relative change in expression day 0: 1 ± 0.54, day14: 4.49 ± 0.89; p<0.001) (Figure 6A,6B). There was a 126% increase in Runx2, a major regulator of osteoblast differentiation (relative change in expression day 0: 1 ± 1.15, day 14: 1.26 ± 0.15; p=0.004), and a 320% increase in TGF-β, a promoter of osteogenesis (relative change in expression day 0: 1 ± 0.11, day 7: 1.61 ± 0.19, day 14: 3.2 ± 0.43; p<0.001) during the 14-day period (Figure 7A,7B).

RANKL mRNA expression presented a slight, non-significant increase (relative change in expression day 0: 1 ± 0.53, day 14: 1.45 ± 0.57; p=0.34) while calponin, an inhibitor of bone formation in osteoblasts, showed a non-significant decrease in its expression (relative change in expression day 0: 1 ± 0.28, day 14: 0.68 ± 0.18; p=0.11) (Figures 8A, 8B).

Discussion

Symptomatic stenosis of CAVD is the most common cause of valve replacement surgery in Europe and North America [1,11]. CAVD has a multifactorial etiology; and its pathogenesis is based on an inflammatory process affecting the valve, which leads to leaflet stiffness due to fibrosis and calcification [12-14]. The process involves deposition of low-density lipoproteins (LDLs) [15,16], osteoblastic transformation with bone formation of valvular interstitial cells, connective tissue synthesis, and tissue remodeling. On a microscopic level, the aortic leaflets contain disorganized collagen fibers, chronic inflammatory cells, extracellular bone matrix proteins, lipidic proteins and bone mineral [17].

The processes of CAVD share many similarities with atherosclerosis, and the pathologies of both conditions have similar risk factors and histopathology [18]. Progressive thickening of the aortic valve leaflets and narrowing of the aortic valve area leads to increased mechanical stress on the left ventricle and reduces cardiac output [12,16,19,20].

The activation of VICs results from cytokines produced by macrophages and T-cells. Mechanical and shear stress leads to endothelial damage and lipid deposition (LDLs), that results in inflammatory events and the presence of inflammatory cells, like monocytes-macrophages and T cells, which produce cytokines such as transforming growth factor beta (TGF-ß), which regulates cell proliferation and differentiation, tumour necrosis factor alfa (TNFα),

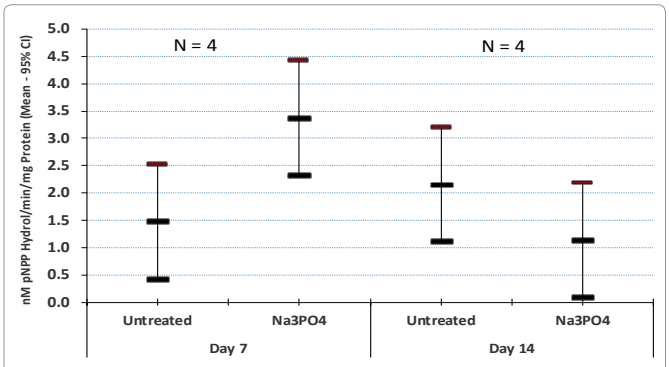

Figure 5: The effect of Na_3PO_4 on ALP activity. ALP activity was increased by day 7 and decreased by day 14 in the Na_3PO_4 treated cells.

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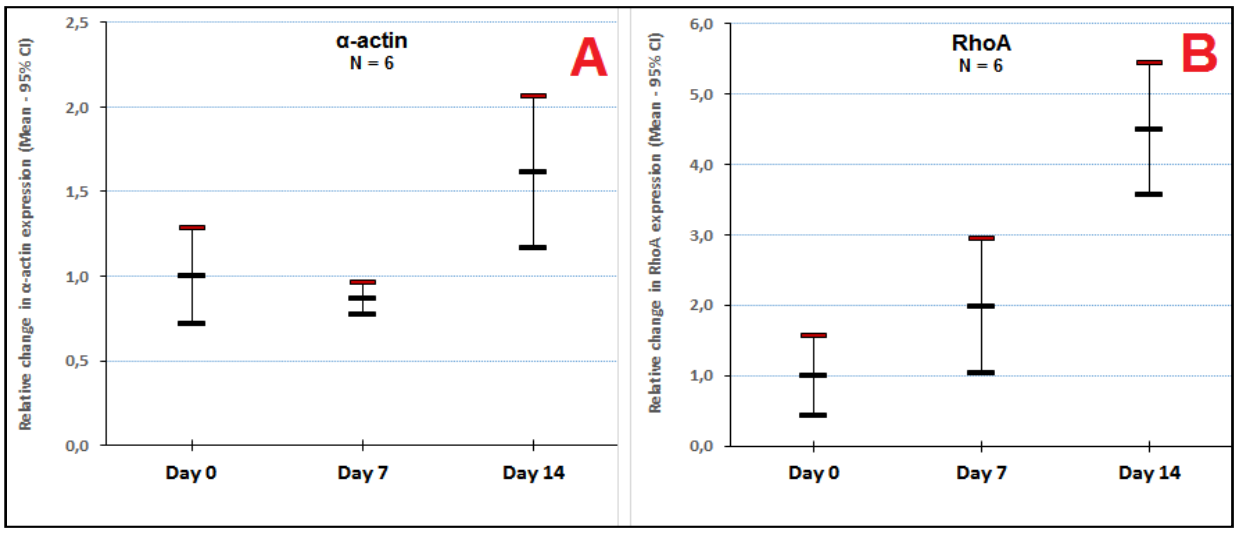

Figure 6: qPCR analysis of porcine VICs during spontaneous calcification. Relative change in α-actin (4A) and RhoA (4B) expression.

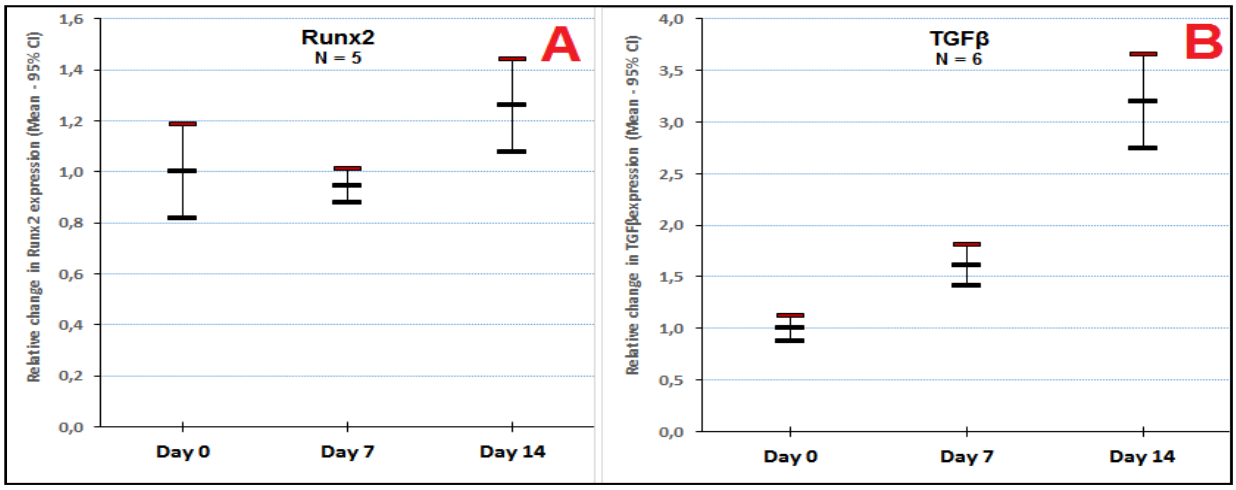

Figure 7: qPCR analysis of porcine VICs during spontaneous calcification. Relative change in Runx2 (5A) and TGF-β (5B) expression.

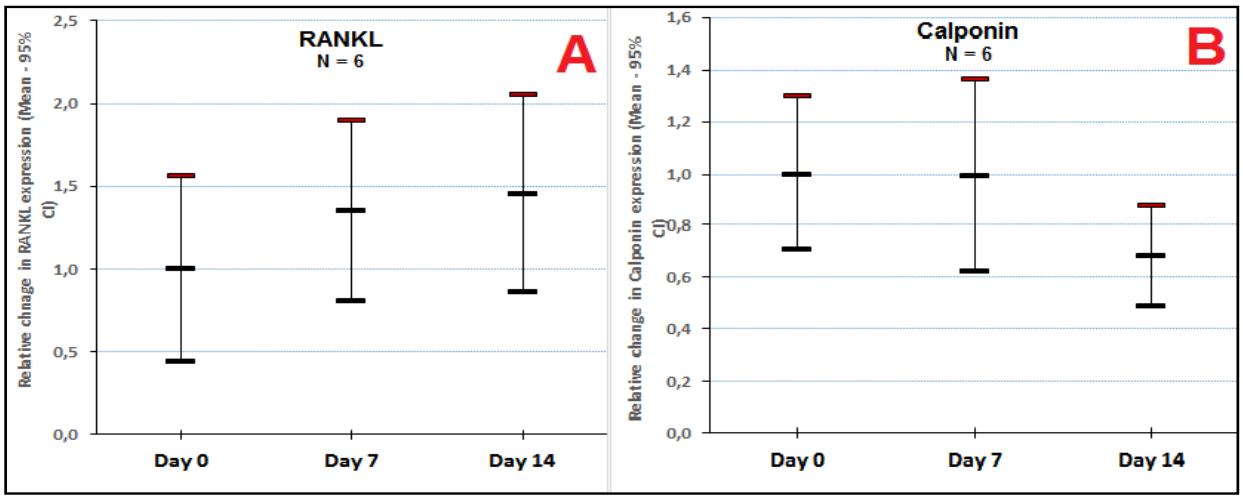

Figure 8: qPCR analysis of porcine VICs during spontaneous calcification. Relative change in RANKL (6A) and Calponin (6B) expression.

whose primary function is the regulation of the immune cells, and interleukin 2, which is produced by activated T-lymphocytes with growth factor activity [1] VICs activated by the inflammatory process are designated myofibroblasts [17]. These cells will develop angiogenic activity and produce matrix metalloproteinases (MMPs), which are involved in tissue remodeling, and play a role in further activation and transformation of VICs [21,22]. During this process, activated VICs differentiate into osteoblast.

During the characterization of porcine aortic VICs, our first objective was to determine the expression level of a common marker of myofibroblast phenotype, α-Actin, in order to demonstrate that active VICs were present in the samples. Next, the expression of Runx2, a major regulator of osteoblast differentiation, was analyzed to corroborate that the effect of the complete trans-differentiation of VICs had taken place, and that the osteoblast phenotype was present. Furthermore, changes in the expression of TGF--β, a promoter of osteogenesis, were detected and recorded. Additionally, RhoA, a regulator of nodule formation in myofibroblasts, was analyzed follow by changes in the expression of RANKL, a key regulator of bone metabolism, which is found on VICs membranes and that is the target of potential inhibitors of calcification. Finally, Calponin, a protein with potential capability to inhibit bone formation, was measured to complete the genetic studies.

Although RANKL relative expression levels remained constant during the trans-differentiation of VICs, it would be useful to further analyze RANK expression changes, as it is the trans-membrane receptor involved in VICs activation. TGF-ß can increase calcium and collagen deposition [23]. It is known that TGF-ß can also stimulate the expression of RANK on pre-osteoclastic cells, and in this way increase osteoclastic sensitivity to RANKL [24]. RANKL is expressed in the membrane of osteoblasts and monocytes.

Porcine aortic VICs have shown excellent response to induced calcification with Na_3PO_4, showing nodular formation and significant increase in ALP activity by day 7 ($P<0.001$) ALP activity which increased significantly by day 7 within the Na_3PO_4 treated group showed a decrease by day 14 but remained elevated in the untreated group of VICs. It could be that the intensification of the calcification process caused by Na_3PO_4 would affect the function of cells producing ALP by the end of the 7 days period.

Conclusions

Porcine VICs are good model to study the process of CAVD. This study has demonstrated the upregulation of some key molecules during the spontaneous calcification of VICs with an increase of calcium, collagen and ALP activity. The cells expressed markers for both vascular smooth muscle cells and osteoblasts, suggesting a transdifferentiation of the phenotype. Upregulation of α-Actin, RhoA, Runx2 and TGF-β were noted, with no changes seen in RANKL or calponin expression. Calcification of VICs was successfully increased by the addition of Na_3PO_4 3 mM (pH 7.4) with a significant escalation of calcium, collagen by day 14, and ALP activity by day 7.

The limitation of our study is that, the extrapolation of the results acquired in an *in vitro* animal model to humans should be done very carefully, as species variations are likely to exist. Although it is impossible to include all mechanisms involved in CAVD in a single experimental model, it can contribute towards determining the role several factors could play in the development of CAVD. A fuller understanding of the intrinsic mechanisms involved in this process may contribute towards the identification of a novel therapeutic strategy for clinical intervention against aortic valve calcification and aortic stenosis.

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