Review Article

Ectopic mineralization in heart valves: new insights from *in vivo* and *in vitro* procalcific models and promising perspectives on noncalcifiable bioengineered valves

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Abstract: Ectopic calcification of native and bioprosthetic heart valves represents a major public health problem causing severe morbidity and mortality worldwide. Valve procalcific degeneration is known to be caused mainly by calcium salt precipitation onto membranes of suffering non-scavenged cells and dead-cell-derived products acting as major hydroxyapatite nucleators. Although etiopathogenesis of calcification in native valves is still far from being exhaustively elucidated, it is well known that bioprosthesis mineralization may be primed by glutaraldehyde-mediated toxicity for xenografts, cryopreservation-related damage for allografts and graft immune rejection for both. Instead, mechanical valves, which are free from calcification, are extremely thrombogenic, requiring chronic anticoagulation therapies for transplanted patients. Since surgical substitution of failed valves is still the leading therapeutic option, progressive improvements in tissue engineering techniques are crucial to attain readily available valve implants with good biocompatibility, proper functionality and long-term durability in order to meet the considerable clinical demand for valve substitutes. Bioengineered valves obtained from acellular non-valvular scaffolds or decellularized native valves are proving to be a compelling alternative to mechanical and bioprosthetic valve implants, as they appear to permit repopulation by the host's own cells with associated tissue remodelling, growth and repair, besides showing less propensity to calcification and adequate hemodynamic performances. In this review, insights into valve calcification onset as revealed by *in vivo* and *in vitro* procalcific models are updated as well as advances in the field of valve bioengineering.

Keywords: Ectopic mineralization; aortic valve calcification; procalcific models; bioprosthetic heart valves; tissue-engineered heart valves.

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Introduction

Anatomically located between the left ventricle outflow tract and the ascending aorta root, aortic valves play a key functional role in the cardiac cycle by enabling blood influx into the aorta lumen in response to ventricular systole and avoiding blood backflow into the left ventricle chamber during ventricular diastole. In such hemodynamics, valve cusps are subjected to recurring cycles of bending, shearing and tearing stresses, since they cyclically open and close about a billion times during a lifetime. It is thus not surprising that these specialized anatomic components frequently undergo structural alterations leading to two distinct congenital or acquired valvulopathies, i.e., aortic valve regurgitation and calcific aortic valve stenosis (CAVS).

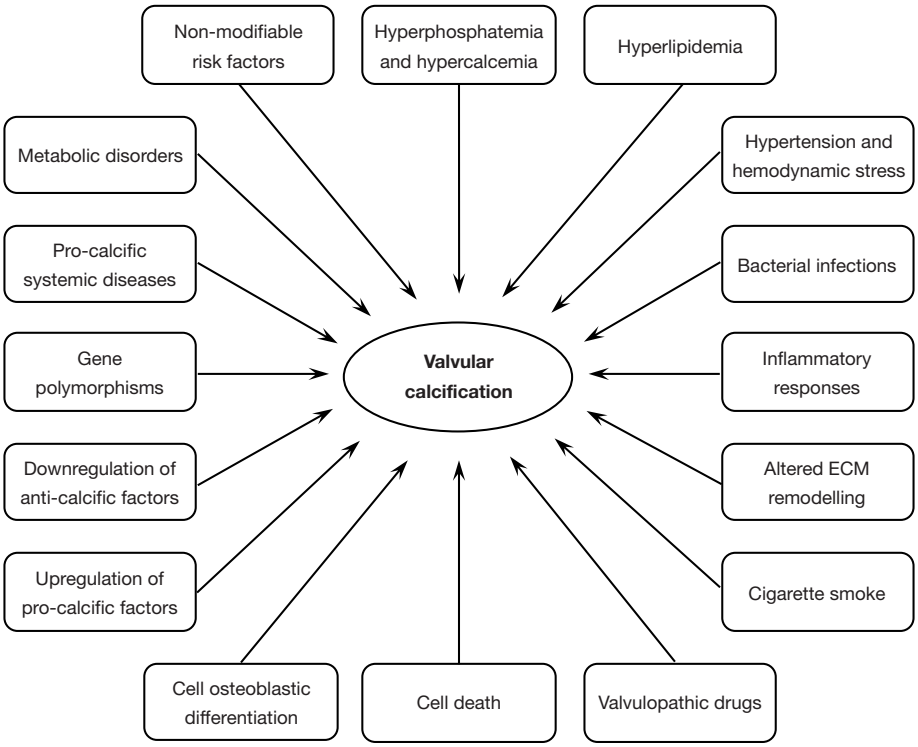

Figure 1 Factors reported to be potentially involved in aortic valve mineralization.

Aortic valve regurgitation is due to cusp weakening commonly associated with dilation of the ascending aorta root, causing improper valve closure with blood re-entry into the left ventricle chamber during ventricular diastole. Conversely, CAVS is characterized by cusp stiffness due to fibrosis and calcification, with obstruction of the left ventricular outflow tract leading to concentric left ventricle hypertrophy with associated angina, syncope and dyspnea (1). Valve calcification is a form of ectopic calcification due to very poor cusp vascularization with the consequent absence of an efficient macrophage-mediated scavenging of degenerated cells and their remnants, which act as early calcification foci. Valve calcification is conventionally distinguished into (I) metastatic calcification, i.e., diffused calcification due to systemic mineral imbalance, typically affecting uremic subjects, and (II) dystrophic calcification, i.e., topic calcification caused by tissue injury, aging and/or existence of other comorbidities, typically occurring in non-uremic subjects (2). Degenerative CAVS is the most common valve disorder caused by valve dystrophic calcification affecting the elderly population in the Western world (3). First described by Mönckeberg in 1904 (4), CAVS was regarded for years as a passive age-related process, with the major role being played by the prolonged shear-and-tear effect of hemodynamic stress on valve cusps (5-7). In the last two decades, the concept of CAVS etiopathogenesis has undergone considerable changes in that this valve disease is no longer regarded as the end stage of a mere degenerative process associated with cell death (8-11), but rather as a multifactorial calcific disorder including (I) accumulation of altered lipids, (II) release of proinflammatory mediators, (III) downregulated expression of anti-calcific factors, (IV) gene polymorphisms and (V) cell osteoblastic differentiation (12-22) (*Figure 1*). Concerning this latter aspect, reports have increased of possible transdifferentiation of aortic valve interstitial cells (AVICs) into osteoblast-like cells with heterotopic bone formation (23-28). Although these interesting results contribute to increasing knowledge on the processes underlying calcific valve disorders, they seem to have introduced the misconception that the terms "ectopic calcification" and "ectopic ossification" indicate the same pathological process. In this regard, it is worth pointing out that heterotopic bone formation was histologically shown to have occurred in less than 13% of thousands of explanted CAVS-affected valves (14,24) and actual osteocyte-like

differentiation was observed in only one of 31 failed heart valves (23), strongly suggesting that valve ossification may be a mere epiphenomenon sometimes co-occurring with or superimposing on valve calcification, rather than this histogenetic process being regarded as a *sine qua non* for the occurrence of mineralizing processes. Ectopic calcification is also the main cause of failure in the mid to long term of bioprosthetic valves fabricated using native valves or pericardium harvested from animals (xenografts) or humans (allografts) (29,30). It was estimated that 20% to 30% of bioprosthetic valves require replacement within 10 years of implantation, with failed valve bioprostheses increasing up to 50%, or more in pediatric patients, within 15 years of implantation (2,29). Malfunctioning of implanted valve bioprostheses is reported to be due mainly to (I) cytotoxic effects exerted by treatments with glutaraldehyde (GA) or other crosslinking agents for xenografts (29,31-33), (II) tissue alterations caused by cryopreservation/thawing for allografts (29,34-37) and (III) occurrence of graft-versus-host rejection for both types (34,35,37-40). Disadvantages limiting the efficacy of the valve substitutes currently used in clinical practice in terms of biocompatibility, durability and capacity for tissue remodelling stimulated the development of novel tissue-engineered heart valves (TEHVs) (41). TEHVs can be obtained using synthetic or biological acellular scaffolds as well as scaffolds deriving from heart valves deprived of their resident cell population, all sharing the condition of being free of chemical treatments with any crosslinking agent. Such innovative bioengineered valves are being shown to permit both *in vitro* and *in vivo* cell repopulation, with good potential for tissue remodelling, adequate biocompatibility, proper mechanical behaviour and reduced propensity to mineralization (42-45), appearing as a promising alternative to the commercially available valve bioprostheses.

Ectopic calcification and cell death

Calcification is a widespread process that occurs physiologically in biological systems, from unicellular organisms to hard tissues belonging to Invertebrates and Vertebrates, besides involving soft tissues affected by various calcific diseases (46). In bones and teeth, major mineral nucleation sites are represented by cell-derived matrix vesicles, the production of which surely depends on distinct, active processes involving osteoblasts and odontoblasts, respectively (47-50). Release of analogous mineralizing matrix vesicles in association with apoptosis activation was also reported for hypertrophic chondrocytes in calcifying cartilages (51-53). In contrast, ectopic calcification in soft tissues is characterized by evident tissue alterations including different types of cell death, which have not yet been unequivocally identified. Despite a number of involved cell pathways having been highlighted, especially concerning apoptotic death, the major underlying problem is that a *mare magnum* exists of terms used to classify, more or less exhaustively, the various forms of cell death, with their mechanisms and pathogenetic factors still being far from a clear elucidation of their nature (54-56). It ensues that characterization of the upstream processes leading to the production of dying-cell-derived byproducts requires more knowledge to clearly distinguish how far and to what extent the so-called "matrix vesicles", "apoptotic bodies", "cell-derived bodies", "membrane vesicle bodies", "membrane blebs", "exosomes" etc. are distinct structures or heteromorphic variants of the same form. In addition to these vesicular degenerative products, which are all cytoplasm-containing, membrane-bounded roundish bodies or polymorphic membranous debris derived from cells or cell organules, peculiar cell byproducts lined by alcianophilic thick walls were ultrastructurally identified in calcifying cardiovascular tissues (57,58). More recently, analogous peculiar structures named "PPL-vesicles" were ultrastructurally found to originate from the surface of pre-calcific dying AVICs after formation of a thick phthalocyanin-positive-layer (PPL) at their edges, as described below (59-65). Since it resulted that the sequential degenerative steps leading to PPL-vesicle release were quite different from those shown by cells undergoing the conventional cell death forms involved in valve calcification onset, i.e., apoptosis (8-10), necrosis (8) or autophagocytosis (11), it was suggested that an additional type of cell death might be associated with valve mineralization, as mentioned below. Further cell byproducts identified in a lot of calcified soft tissues, including native and bioprosthetic heart valves, consist of concentrically arranged, multi-laminated vesicular bodies that were named *spherulites* or *calcospherulae* (8,66-69). These structures appeared to act as early hydroxyapatite (HA) nucleators, with their electrondense layers representing an optimum substrate for the paracrystallin precipitation of radially oriented crystallites, which, in their turn, may promote additional autocatalytic mineral deposition involving wider and wider tissue areas. It is also of interest that synthetic vesicles ultrastructurally showing multilamellar envelopes made of lipid (70,71) or protein (72) anionic molecules

were found to act as strong HA nucleators in *in vitro* environments.

Ectopic calcification, polyanions and cationic copper phthalocyanins

In the 1970s, lipid material accumulation was found to be strictly associated with increased precipitation of HA crystals in mineralizing hard and soft tissues in both physiological and pathological calcification processes. In particular, cell-membrane-derived acidic phospholipids forming the so-called "calcium-phospholipid-phosphate complexes" were originally identified in calcifying cartilage (73) and bone (74). Later, involvement of analogous anionic HA-nucleating complexes was found for pathological calcifications affecting soft tissues including vascular ones (75,76), in which additional identification of proteolipids suggested their contribution to ectopic mineralization (77,78). Moreover, Raman analysis of calcified human aortic valves and atherosclerotic plaques revealed carotenoids to be another lipid moiety that may play a role in pathological calcification of cardiovascular tissues (79-81). Consistently, lipid extraction from valve tissues before being subjected to experimental calcification was found to drastically reduce tissue mineralization (82,83). Similarly, lipid extraction from calcified valves was associated with complete removal of PPLs, revealing this lipid material to be the main substrate for HA crystal precipitation in aortic valves subjected to experimental calcification (59), as described below. Interestingly, lipid substances accumulating in calcified valve tissues were often found to undergo oxidation, so acquiring increased affinity for calcium ions (13,22,84). Consistently with their anionic nature, also ossification-related calcium-binding proteins, such as osteocalcin (16,23,85), osteopontin (16,23,86,87) and osteonectin (88), as well as proteoglycans rich in acidic glycosaminoglycan lateral chains (89-93) were reported to be involved in cardiovascular tissue calcification. In the past, copper phthalocyanins, such as alcian blue, cuprolinic blue (CB) and cupromeronic blue, proved to be the most effective histochemical reagents for the evidentiation of all types of polyanions, including extracellular glycosaminoglycans/proteoglycans (*Figure 2A*,*B*) and intracellular nucleic acids, in both light (94,95) and electron (96-98) microscopy. In early ultrastructural studies on calcified aortic valves and aorta walls, these cationic reagents revealed the presence of alcianophilic material at the surface of cells and cell debris, which was assumed to be formed by proteoglycans mixed with membrane-derived phospholipids (57,58). These fragmentary findings were later found to represent the latest intracellular stage of a multistep process, which was described after using phthalocyanins in ultrastructural studies on aortic valves or cultured AVICs subjected to experimental models of calcification (59-65). In particular, this procedural approach enabled an answer to be given to the following issues: (I) how the phthalocyanin-reactive acidic material is generated, (II) whether it actually represents a major HA nucleator and (III) what relationship exists between the phthalocyanin-reactive material and the extracellular structures actively or passively involved in calcification, such as collagen fibrils, elastin fibers and cell-derived *calcospherulae*. As a first step, the standard phthalocyanin-based staining procedure was modified by dissolving CB dye in an acidulated buffer (pH 4.8) to enable gentle unmasking of the phthalocyanin-positive material from superimposed HA crystallites concurrently with its *in situ* retention and staining. This modified histochemical technique was first applied on porcine aortic valves subjected to *in vivo* calcification induction using an animal model of xenogeneic valve subdermal implantation (59-62). In this context, peculiar PPLs were found to originate from colliquation of all cell membranous components, with intracellular release of an acidic amorphous material (PPM) and its subsequent centrifugal spreading and layering at the edges of dying AVICs (*Figure 2C*). Further release of PPL-vesicles due to blebbing of superficial PPLs was found to occur during later degenerative stages. Consistently with such a peculiar procalcific process, PPLs resulted as being formed mainly by acidic phospholipids, as revealed by different susceptibility to extraction and digestion procedures as well as positivity to suitably modified malachite-green-based histochemical techniques targeted at the identification of these lipids. Peripheral PPLs were found to play a major procalcific role, as revealed by massive clustering of HA crystals at the edges of cells and cell-derived debris in undecalcified samples as well as superimposed precipitation of silver particles onto surface PPLs in decalcified samples additionally subjected to a modified post-embedding von Kossa reaction suitably adapted for the ultrastructural evidencing of calcium-binding sites. Likewise, close association between PPL appearance and increases in calcium amounts estimated spectrophotometrically in parallel samples supported the evidence that a major procalcific role is played by PPLs during experimental valve calcification. The cell-derived PPL-vesicles observed were

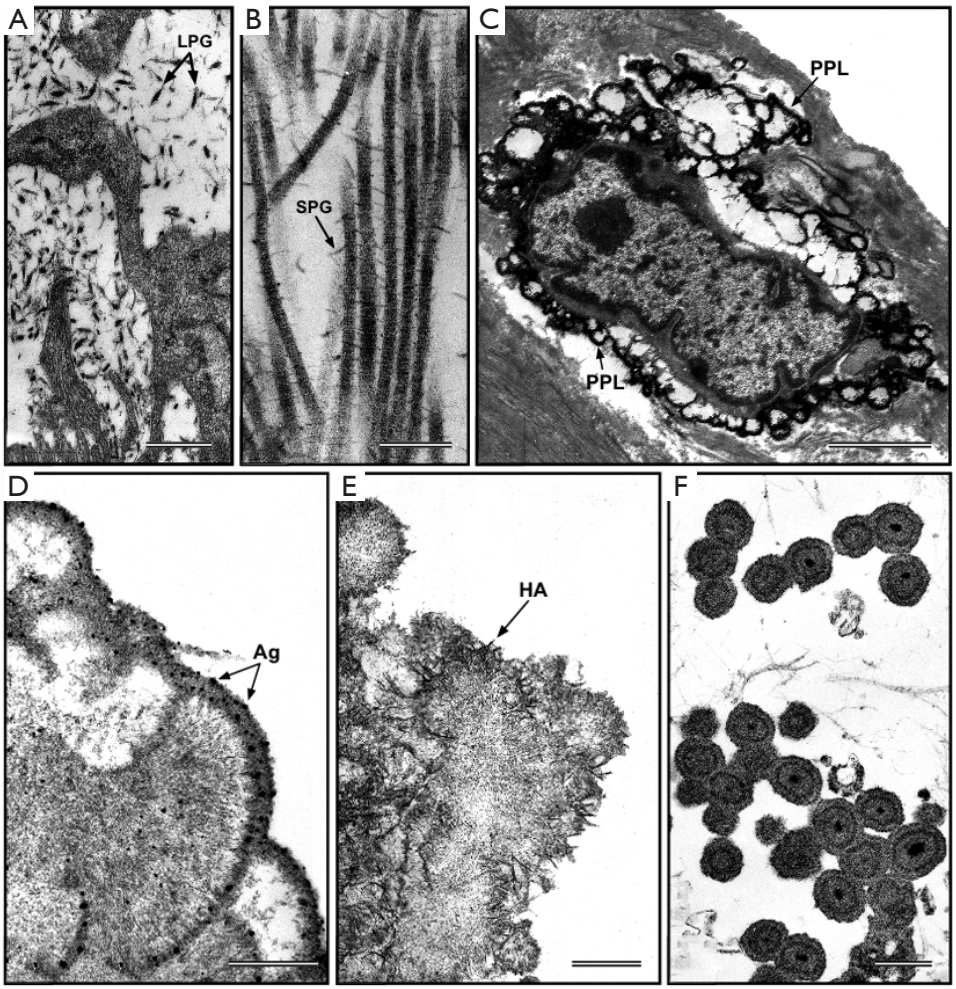

Figure 2 Ultrastructural visualization of polyanionic compounds after pre-embedding histochemical reaction with phthalocyanin cuprolinic blue (CB). (A) CB-positive interstitial leaf-like large proteoglycans (LPG) and (B) CB-positive rod-like small proteoglycans (SPG) interconnecting adjacent collagen fibrils, in native aortic valve cusps. (C) Phthalocyanin-positive layer (PPL) edging a calcifying interstitial cell in an aortic valve cusp subjected to *in vivo* experimental calcification. (D) Peripheral PPL showing superimposed silver particles (Ag) after additional post-embedding von Kossa reaction in a degenerating aortic valve interstitial cell (AVIC) subjected to *in vitro* calcification. (E) Peripheral precipitation of needle-like hydroxyapatite (HA) crystals at the level of underlying PPL in a degenerating AVIC subjected to *in vitro* calcification. (F) PPL-lined calcospherulae originated from vesicular remnants released by AVICs subjected to *in vitro* calcification. Bar: 0.5 μm (A); 0.25 μm (B); 25 μm (C); 0.5 μm (D); 0.5 μm (E); 1 μm (F).

barely reminiscent of matrix vesicles because they resulted from cell blebbing, showed vesicular features and played an analogous procalcific role. However, they also appeared to be quite different from matrix vesicles, since they were edged by 120- to 600-nm-thick multilamellar PPLs instead of the orthodox 7-nm-thick bileaflet cell membrane and seemed not to contain cytoplasmic material. Moreover, a relationship was found to exist between such PPL-vesicles and interstitial *calcospherulae*, with the former clearly showing further rearrangement of the PPL substance into two to four irregularly spaced, concentric rings sometimes encircling an electrondense core. It is of note that extracellular matrix calcification was found to occur once collagen fibrils and elastin fibers were embedded by PPL material spreading from adjacent dead cells or PPL-vesicles, suggesting that valve mineralization is mainly primed by the procalcific degeneration of resident cells with just a secondary involvement of degenerating extracellular matrix

components, as previously reported (99-101).

In vivo and in vitro experimental calcification of aortic valves

To shed light on the etiopathogenesis of heart valve calcification, procalcific animal models were developed, consisting of valve replacement in sheep and calves (102-105) or valve cusp subcutaneous implantation in rabbits or rats (104,106-108). Implanted valves and cusps were found to undergo mineralization showing histopathological features comparable to those of failed valve implants in humans, but with more accelerated kinetics (3 to 6 months for circulatory models and about 8 weeks for subcutaneous models), making such animal models also suitable for investigating the feasibility and effectiveness of potential anti-calcification strategies (109-111). In particular, using *in vivo* models of xenogeneic implantation of porcine aortic valve cusps into subdermal pouches surgically prepared in young rats, the main event in valve mineralization consisted of HA crystal precipitation at the level of roundish structures released by non-viable cells, which were initially described as matrix-vesicle-like bodies (99,104,107,112). Devitalization of valvular cells due to cusp fixation with 0.6% GA resulted as a prerequisite for valve mineralization occurrence, supporting the idea that the use of this chemical treatment for manufacturing of valve bioprostheses may be the most relevant cause of their failure (113). Consistently, non-fixed or alternatively processed valve cusps subjected to *in vivo* implantation were found to be free of mineralization (114-117). After using the rat subcutaneous implantation model, ultrastructural examination of explanted valve cusps subjected to pre-embedding CB-based histochemical reactions as described above revealed the presence of electrondense, lipid-rich PPLs layered at the periphery of degenerating cells (*Figure 2C*), which showed blebbing features and were involved in the formation of roundish cell-derived PPL-vesicles (59-61). Systematic ultrastructural studies on valve cusps explanted after increasing implantation times allowed the entire sequence of degenerative steps underlying the onset and progression of valve calcification to be defined (62), revealing the occurrence of a peculiar type of procalcific cell death characterized by a fast, dramatic breakdown of all cell membranous components, culminating with the detachment from dying AVICs of PPL-vesicles promoting subsequent calcification of the surrounding extracellular matrix, as described above. In the last two decades, several *in vitro* models were also developed to simulate etiopathogenetic environments promoting cell mineralization (118-122). Namely, smooth muscle cells or AVICs were cultured in media containing inorganic phosphate (Pi) at high concentrations (Pi ≥2.0 mM) and/or proinflammatory mediators that were expected to increase cell susceptibility to calcification. More recently, analogous procalcific *in vitro* models were set up, in which primary cultures of bovine AVICs were treated with different combinations of hyperphosphatemic- or normophosphatemic-like Pi concentrations, bacterial lipopolysaccharide (LPS) and conditioned medium obtained from cultures of LPS-stimulated macrophages (63-65). Despite identification of AVIC subtypes particularly prone to mineralization having been reported (122-124), entire AVIC populations were stimulated in these *in vitro* models in order to achieve a more faithful reproduction of native conditions. Ultrastructural analyses paralleled by spectrophotometric measurements of calcium amounts and alkaline phosphate activity supported the concept that cell exposure to high Pi concentrations is a prerequisite for priming AVIC mineralization, which was exacerbated by cell superstimulation with proinflammatory mediators (63,65). Using the CB-based histochemical procedures as above, also cultured AVICs appeared to undergo the same procalcific degeneration as that previously described for AVICs populating aortic valves subjected to *in vivo* calcification induction (*Table 1*), including accumulation of intracellular PPM, appearance of surface pro-calcific PPLs (*Figure 2D* and 2E), release of PPL-vesicles and their subsequent transformation into *calcospherulae* (*Figure 2F*). More information on cell response to non-calcific or sub-calcific environments was achieved by treating AVICs with different Pi concentrations like those spanning the normal range in organisms (65). Interestingly, two opposing Pi-dose-dependent cell responses were found to be evoked, i.e., cell survival *versus* procalcific cell death (*Figure 3*). Namely, AVICs exposed to low/medium Pi concentrations (0.8 and 1.3 mM) were found to undergo atypical autophagic activity in which a particularly hypertrophic endoplasmic reticulum appeared to be directly engaged in organelle sequestration and digestion, consistently with parallel time-dependent decreasing expression of autophagocytosis-related lysosomal markers. Since neither cells immunopositive for apoptosis markers nor cells showing degenerative features at ultrastructural level were encountered, the concept was supported that the observed non-lysosomal autophagic activity may correlate with AVIC survival. Conversely, AVICs treated with the highest Pi concentration (2.0

Table 1 Progressive stages of AVIC deterioration in *in vitro* calcification as revealed by transmission electron microscopy

Degenerative features	3 days	9 days	15 days	21 days	28 days
Organelle disappearance	–	±	+/++	++	+++
PPM appearance	–	–	++	+	–
PPL appearance	–	–	±	++	+++
EB-CS appearance	–	–	–	+	++
HA appearance	–	–	–	+	+++

AVIC, aortic valve interstitial cell; PPM, intracellular degradation-derived phthalocyanin-positive material; PPL, PPM-derived pericellular phthalocyanin-positive layers; EB-CS, PPL-derived extracellular blebs plus their subsequent byproducts identifiable as real calcospherulae; HA, needle-like hydroxyapatite crystals.

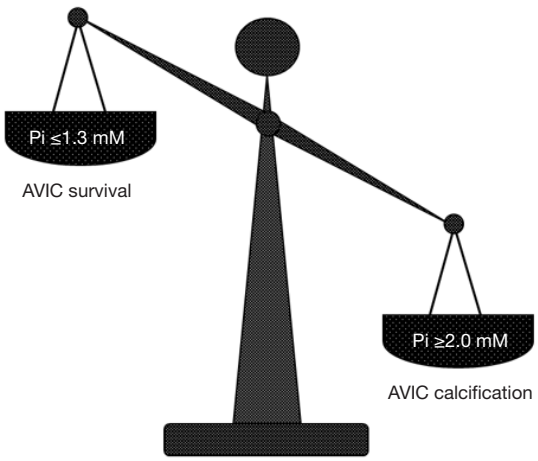

Figure 3 Critical inorganic phosphate (Pi) concentrations driving the fate of cultured aortic valve interstitial cells (AVICs).

mM) were found to undergo mineralization following the same steps as those in the above-described lipid-release-associated procalcific cell death, supporting the concept that individuals with high normophosphatemic values are at increased risk of valve dystrophic mineralization. Since the *in vitro* models used allowed a reliable reproduction of AVIC calcification, including the genesis of *calcospherulae* as those observed in pathologically calcified heart valves (8,66,68), they could be usefully exploited on the one hand to find the molecular mechanisms associated with this type of cell death and on the other to assay the pro- or anti-calcific effects exerted on AVICs by putative stimulating or inhibitory agents, respectively.

Tissue-engineered heart valves

To date, surgical substitution of failed heart valves remains the leading therapeutic option for patients affected by valvular diseases (2,125). In the 1960s, mechanical valves were the first substitutes to be introduced in clinical practice, the Starr-Edwards caged-ball valve, Bjork-Shiley and Hall-Medtronic tilting-disk valves and St. Jude Medical bileaflet tilting-disk valve being the most widely used (29). Being prepared using synthetic materials such as titanium, cobalt-chromium alloy or pyrolytic carbon, mechanical implants have proven to be highly thrombogenic (126-133), requiring chronic anticoagulation therapies for transplanted patients and thus excluding their use in women of childbearing age (134-137). A further disadvantage is that implantation of mechanical valves in children requires reoperation in the medium term because these substitutes are unable to increase in size with the patient's growth (138,139). Despite having shorter durability than mechanical devices, valve bioprostheses rapidly became widespread substitutes because of their native-like valve shape enabling better graft thromboresistance and adaptation to the hemodynamic flux (140-143). Bioprosthetic valves include (I) xenografts, which are usually fabricated using porcine valve cusps or bovine pericardium-derived limbs mounted (stent) or not (stentless) on a prosthetic metal frame and (II) allografts manufactured using human pericardium limbs or consisting of cryopreserved aortic valves harvested from human cadavers or "fresh" aortic valves excised from beating donor hearts at transplantation (29). Valve xenografts, such as the Hancock valve and Carpentier-Edwards valve, are prepared using animal tissues conventionally subjected to chemical crosslinking with GA, so allowing tissue sterilization and suppression of tissue immunogenicity due to retention of resident cells. Regrettably, such chemical fixation proved to be the major cause of xenograft failure because of GA-dependent cytotoxic effects and concurrent defective graft immunosuppression. Indeed, valve xenografts were found

to be characterized by an absence of viable cells, besides showing calcific foci mainly at the level of the *tunica spongiosa* and cuspal commissures (31-33,107,144-146), with calcific deposits co-localizing with cell remnants, including interstitial *calcospherulae*, and collagen fibrils, as revealed ultrastructurally (66,68,69,147-152). Compared to xenografts, both cryopreserved and "fresh" valve allografts are expected to be better devices and, indeed, their slightly longer durability has been reported (153-159). However, these valve substitutes also showed some inconveniences such as (I) limited availability, (II) poor suitability for transplantation in pediatric patients (160-162) and, above all, (III) propensity to mineralization (35-37,163-168). Although no treatments with crosslinking agents are applied, sterilization procedures, cryopreservation-related damage and graft-versus-host rejection are suspected to contribute, alone or together, to allograft procalcific degeneration (34,169-174). Despite improvements being reported introducing alternative GA-free tissue treatments for xenografts (114-117,175-179) or less harmful cryopreservation and thawing procedures for allografts (180,181), tissue decellularization seems to be the most promising procedure to obtain biocompatible TEHVs free of adverse immune responses and calcification, being concurrently permissive of suitable cell repopulation, capable of tissue growth and remodelling and having adequate hemodynamic properties. In particular, detergent-based protocols utilizing SDS or sodium deoxycholate, also combined with Triton X-100, were found to be particularly suitable for tissue decellularization, providing acellular scaffolds with a well-preserved extracellular matrix texture and proper biomechanical behaviour, besides showing reduced susceptibility to tissue mineralization (182-187). Removal of resident cells with such decellularizing procedures offered the additional advantage of eliminating cell-associated antigens, which are responsible for the immunogenicity of valve bioprostheses including xenografts despite their chemical treatment with GA (188-190). Consistently, early failure of commercially available decellularized xenografts (SynerGraft, Matrix P) implanted in patients seemed to be ascribable to the use of defective cell removal procedures with persistence of cell remnants within valvular tissues (191,192). Conversely, clinical implantation of commercial decellularized-cryopreserved allografts (SynerGraft, CryoValve) gave very encouraging outcomes in terms of reduced antigenicity, retained structural integrity and long-term durability (193-199), although their clinical application is still hampered by their limited availability. First attempts to attain functional TEHVs were performed *in vitro* by statically seeding autologous or heterologous stem cells or differentiated cells on acellular synthetic or biological scaffolds as well as decellularized valve cusps. These constructs were found to undergo an almost complete re-endothelialization as well as side-by-side repopulation by seeded cells that acquired phenotypical features like those in native aortic valves (185,200-211). Subsequent use of bioreactors to test the resistance and hemodynamic behavior of repopulated scaffolds provided evidence that mechanical stresses stimulate the metabolic activity of entered cells with enhanced extracellular matrix remodelling (212-220). More recently, circulatory animal models have been preferred to dynamic bioreactors because they allow more reliable functional properties of implanted valve scaffolds to be obtained (221-226). Apart from non-human primates, pigs, rather than calves, dogs and the most often used sheep, have proved to be the animals that better replicate the anatomical and physiological features of human cardiovascular apparatus (227), including coagulation mechanisms and inflammatory system response (228). In particular, Vietnamese pigs appear to be an optimal choice to test the long-term follow up of implanted TEHVs because these miniature swine provide the additional advantages of (I) heart rate, cardiac stroke volume, mean arterial pressure and myocardial blood flow that almost coincide with those in humans (229) and (II) growth rates that are comparable to those of pediatric patients (230). Indeed, TEHVs implanted in mini-pigs were found to be suitably repopulated by native-like cells, with associated tissue growth and remodelling, besides showing good hemodynamic performances and limited tissue alterations even in the long-term follow up (231-234). The Vietnamese pig model was also used to test valve implants after cryopreservation and thawing, prefiguring a hypothetical scenario where cell removal from heart valves could become a standard procedure before their storage in valve cryobanks. Although these decellularized and cryopreserved porcine valves showed an acceptable functional activity, their structural features fell below expectations when histological and ultrastructural examinations revealed valve implants to be suitably covered by monolayered endothelium-like cells and populated by entered interstitial cells only in restricted cusp areas (235). These undesirable defects were reasonably ascribed to a sub-optimal preservation of graft extracellular matrix, with reduced propensity by host cells to enter, survive and properly differentiate. Once pre-implant

valve scaffold cryopreservation procedures have been improved, cryobanks could be created of readily available, decellularized valve scaffolds capable of proper post-implant regeneration in order to meet the clinical demand for revitalizable pseudo-autologous valve substitutes.

Concluding remarks

Thanks to their morpho-functional features, heart valves enable a proper incessant cardiac cycle over an entire lifetime, withstanding the intermittent mechanical stresses due to hemodynamic pressure changes and blood flow friction. Accordingly, their malfunctions or breakdown relentlessly lead to life-threatening conditions. Of these anatomical elements, the aortic valves are the most commonly affected by calcification-associated disorders, with substantial improvements still being needed in predicting propensity to mineralization, providing effective drug therapies or designing surgical approaches to valve transplantation. The concept that calcific events affecting both native valves and bioprosthetic substitutes result from stress-dependent passive valve deterioration is being increasingly overtaken by an antithetical view that such a pathology depends on active processes involving valvular cells. Indeed, new findings are continually shedding light on possible mechanisms leading to valve mineralization even if its etiological triggers are still far from being exhaustively elucidated. Similarly, how much the calcific disease may be regarded as an intra-valve osteogenic process or rather a result of diffuse valve cell suffering compromising survival programs and/or leading to certain types of procalcific death has not yet been defined. Hence, additional investigation is required to shed light on inherent upstream regulatory mechanisms leading to ectopic mineralization. The existence of a specific sequence of procalcific intracellular and extracellular events underlying the calcific phenomenology was revealed ultrastructurally using adapted CB-based techniques in *in vivo* and *in vitro* models of valve calcification, as emphasized above, which may represent a trailblazer for better understanding this type of ectopic mineralization. Concerning surgical valve transplants, the most promising substitutes seem to be TEHVs because of their propensity to suitable cell repopulation, being free of undesirable calcific effects. In order to adopt personalized therapeutic approaches, proper pre-implant *in vitro* colonization of decellularized xenografts or, even better, allografts by seeded host-derived cells would be a time-consuming and complicated procedure. Instead, post-implant spontaneous *in vivo* cell repopulation of acellular scaffolds could be viewed as the best procedural choice also for their availability, once decellularized aortic valves can be methodically stored in cryobanks. Envisaging such a situation, preliminary investigation yielded results which, although not being entirely favourable, surely proved that decellularized and cryopreserved TEHVs do not oppose coating by endothelium-like cells and entering valve scaffolds by interstitial-like cells. In the light of such results, ongoing investigations providing concrete outcomes can be predicted with a certain optimism. In conclusion, although more than a few unresolved issues still persist, increased understanding on pathogenesis, onset and progression of valve calcification seems to be imminent as well as substantial improvements in the achievement of noncalcifiable pseudo-autologous TEHVs capable of the best functional performances in addition to longer durability and, possibly, post-operative growth.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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