# Cellular biology of cryopreserved allograft valves

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**Abstract: Although analyzing the precise mechanisms of cryopreserved allograft valve failure may be difficult due to a number of crucial reasons and the interrelationships between the overlapping mechanisms, there is some evidence that cryopreservation is currently the best method of storing allograft valves. The present review shows the basic cellular biology of cryopreserved allograft valves for long-term durability, particularly relevant to allograft valve cellular viability, the immune response mainly caused by viable donor cells, and the preservation and regeneration of the intrinsic extracellular matrix. The present findings are as follows. First, cryopreservation produces serious damage to cytosolic and mitochondrial functions of both endothelial cells and fibroblasts, which may cause valve failure after implantation. Second, although the collagen synthesis of cryopreserved valves was relatively maintained, total protein synthesis was highly diminished and the collagenolytic ability was activated immediately after the thawing process. These findings imply that the cryopreservation itself may cause the collagen metabolism to become degradable, which will lead to valve failure. Further examination of collagen metabolism and modulation of the collagenolytic activity will be necessary to improve the tissue preservation for improved clinical use.** **J. Med. Invest. 48 : 123-132, 2001**

***Keywords*** : *cryopreservation ; allograft valve ; cellular viability ; collagen synthesis ; collagenolysis ; matrix metalloproteinase*

## INTRODUCTION

Since the first orthotopic implantation of an allograft aortic valve more than 30 years ago (1-4), allograft aortic and pulmonary valves have been increasingly used in the surgical management of aortic valve and aortic root disease (5-8), as well as for the correction of complex congenital heart disease (9-15), and their usefulness is not deniable. Although early allograft valves were variably sterilized in ethylene oxide, preserved in β-propriolactone, irradiated, or freeze-dried, subsequent developments in preservation techniques including antibiotic use (16), improved culture media, and freezing have led to the use of cryopreserved allograft valves. The most common cryopreservation method involves freezing in dimethyl sulfoxide and storage with liquid nitrogen (16).

Although aortic valve replacement with cryopreserved allograft valves achieves excellent early results, eventual failure of these tissues is common (17-20). Despite long-standing and widespread use of cryopreserved allograft valves, the basic cellular biology of these tissues is unknown. Ongoing particular interests and controversies concern allograft valve cellular viability (21-27), the contribution of the immune response mainly caused by viable donor cells (28, 29), and the preservation and regeneration of intrinsic extracellular matrix. It is unclear how may these factors may be associated with valve durability and competence. The present study was undertaken to review the biology of cryopreserved valves relevant to long-term durability and "better" preservation techniques.

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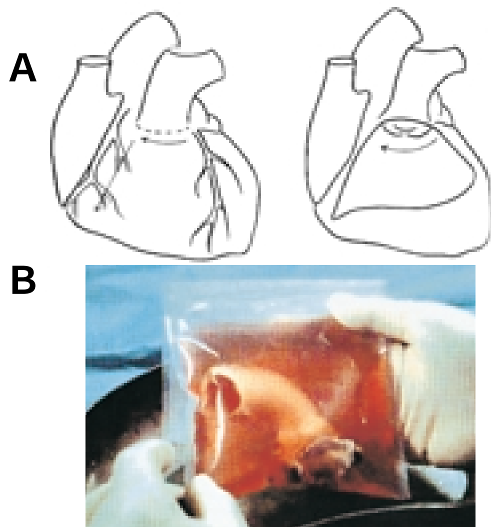


**Fig. 1. Procurement, sterilization, and packaging of pulmonary valve.**
**A: The pulmonary valve is dissected and procured within one hour after death.**
**B: After antibiotic sterilizing treatment (240 μg/ml cefazolin, 120 μg/ml lincomycin, 50 μg/ml vancomycin, and100 μg/ml polymixin B) at 4°C for 24 hours, valves were transferred to TC199 culture medium with 5% N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer, 10% calf serum, and 10% dimethyl sulfoxide.**

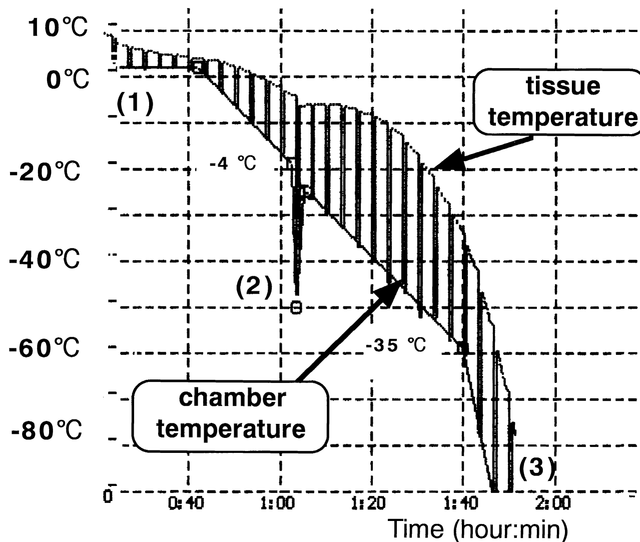


**Fig. 2. Computer-controlled freezing curve.**
**In our institute, the heart valve is frozen with a programmable freezer (Cryomed model 1050, Forma Scientific Co, Marietta, Ohio). Solid line shows the temperature within the chamber. Dotted line shows the temperature of the tissue.**
**(1) Temperature within the chamber is kept at 4°C.**
**(2) Released heat associated with water crystallization around -20°C- -30°C is regulated.**
**(3) Freeze the sample to -80°C (-1°C/min).**

## The most common cryopreservation method

Cryopreservation techniques include four processes as follows: first, sterilization; second, freezing; third, storage; and fourth, thawing.

1) *Sterilization*: Presently, the most commonly used and widely accepted method for allograft tissues is broad-spectrum antibiotics in a nutrient medium (Fig. 1).
2) *Freezing*: Freezing rate of -1°C per minute would be best to maximize fibroblast viability (30-32) (Fig. 2). Freezing is done kinetically in the presence of a cryoprotectant such as glycerol or dimethyl sulfoxide. As the allograft temperature approaches -20°C, most of extracellular water has frozen, and the remaining freezing medium has begun to solidify. The release of heat associated with water crystallization rapidly diminishes at this point. To maintain a consistent -1°C per minute freezing rate, the chamber must be rewarmed to temperatures just below that of the allografts. From this point, the temperature, which declines within the chamber, is directly reflected in parallel with the temperature decline of the allograft tissue.
3) *Storage*: A convenient storage system is the vapor phase of a liquid nitrogen freezer (-196°C).
4) *Thawing*: Fast warming is usually desirable and has been recommended with heart valves. The actual achievable warming rate is dependent upon the total volume of the graft and solution, the insulating qualities of the packaging, and temperature of the thawing medium. Thawing time is commonly 12-14 min (~15°C/min) for aortic and pulmonary valued conduits with a total combined volume of 100 ml for valve and medium.

## Cellular viability of cryopreserved valves

The rarity of valve tissue degeneration of pulmonary autografts and the demonstration that these autografts can grow does suggest that autogenous valve tissue with intact endothelial and fibroblast cells has distinct advantages over allograft valve tissue (33). Several studies suggested that allograft valve viability associated with cryopreservation techniques was recognized as one of the most influencing factors on the long-term durability of cryopreserved allograft valves (34, 35).

Questions of cryopreservation techniques are particularly relevant in addressing allograft valve viability and the ability to repair leaflet wear and tear injury. It is unclear whether viable donor cells, including intrinsic cuspal interstitial tissue cells, mainly consisting of fibroblasts, and endothelial cells, are present at the time of implantation of cryopreserved allograft valves and whether they persist over the long term. In addition, it is unknown if long-term hemodynamic competence of implanted cryopreserved allograft valves requires donor cell viability and the regeneration of the intrinsic extracellular matrix.

Hillbert *et al*. reported that apoptosis occurred in endothelial cells and cuspal interstitial tissue cells of implanted cryopreserved allograft valves, and this might lead to their loss of cellularity (36). This apoptosis may be related to various factors, including immunologic and chemical injury, and hypoxia during valve processing and reperfusion injury at the time of implantation. Whether the apoptosis and acellularity or patchy cellularity commonly observed in clinically explanted allograft valves is due to cryopreservation techniques themselves remains to be determined (37).

*1) Fibroblasts viability*

If the cell viability in allograft aortic valves is crucial in determining the long-term fate of the valves, then the specific cell population of the greatest importance is likely to be the fibroblasts. These cells are by far the most abundant in aortic valves and are responsible for protein synthesis and structural integrity. Prolonged valve competence and durability of cryopreserved valves have been suggested to be derived from the viability of the donor fibroblasts, which can remodel the matrix assembly (28, 29).

VanderKamp *et al*. (30-32) demonstrated that a constant -1°C per minute freezing would be the best rate to maximize the fibroblast viability, and this theory was adopted and has been used as a freezing standard by the clinical tissue bank (e.g. : Cryolife, Inc., Marietta, Ga.). For heart valves, the presence of a high percentage of the fibroblasts which are capable of resynthesizing the collagenous matrix of the valve, as well as maintaining mechanical integrity, is the primary consideration (31, 38, 39).

The viability of any tissue after cryopreservation was suggested to be dependent, in part, upon handling during procurement and prefreezing storage (22-25). Any exposure to non-physiological conditions, such as ischemia, hypoxia, or anoxia causes direct toxicity to most cell types, with subsequent stresses of freezing and thawing (27). Although previous examinations of allograft aortic valves have successfully demonstrated the presence or absence of endothelial cell viability *in vivo*, viability of the fibroblast population has not been demonstrated except in an *in vitro* model (37). Unlike the endothelial cells, whose relatively rapid rate of replication allows convenient methods of assessing their viability, the fibroblast cells replicate slowly and less predictably. Consequently, the evaluation of *in vivo* fibroblast viability requires a method of study that is based on normal metabolic activities of the cell.

There are some experimental studies that favor persistence of donor fibroblast viability after implantation (21-25). Niwaya *et al*. reported that fibroblast viability of the cryopreserved human allograft valve was well preserved (>70%) with a warm ischemic time of less than 520 minutes by flow cytometry (24). Fibroblasts exist within the noncellular matrix, which is composed primarily of collagen, elastin, and proteoglycans (40). Collagen represents the largest portion of the extracellular matrix in the aortic valve. Maintenance of the normal collagen synthesis may be important for the preservation of normal tissue structure and function. If the cellular viability of the fibroblasts helps to determine the capacity of the graft to resist deterioration, this may be mediated by collagen production. Lupinetti *et al*. demonstrated that allografts retain a persistent capacity for procollagen synthesis of fibroblasts similar to that of the native aortic valve (41, 42). However, some clinical studies did not favor donor fibroblast viability after implantation (37). Although persistence of donor fibroblasts has been demonstrated, the histological finding of explanted cryopreserved allograft valves, including allograft valves explanted for reasons other than degeneration, showed acellularity or rare cellularity or patchy cellularity of leaflets (37). The demonstration that fibroblasts of homogenized recently cryopreserved aortic valve tissue can incorporate tritiated glycine

into collagen after short-term implantation (21, 43) does not necessarily translate into the long-term postimplantation ability to repair and regenerate leaflet structure, which is implied by the term "viable".

Our previous study demonstrated that the cytosolic function of cusps, mainly consisting of fibroblasts, was comparatively preserved ; however, the mitochondrial function was still more diminished during these processes (44). These findings imply that cryopreservation causes a latent injury even in fibroblasts, which may cause valve failure after implantation. Lu *et al*. also reported that the mitochondrial dehydrogenase activity of the porcine valve was significantly diminished after cryopreservation processing (45).

*2) Endothelial cell viability and immunogenicity*

The importance of endothelial cells to the long-term fate of allograft valves is unknown. Functions of the vascular endothelium include resistance to thrombosis, maintenance of hemostasis, and modulation of vascular smooth muscle activity. Mediation of immunologic and inflammatory responses is another function of endothelium that is particularly important for allograft valves (46). The vascular endothelium constitutively expresses Class I antigen and is induced to express Class II antigens when it is exposed to an allogenic milieu (47-49). Accordingly, vascular endothelium is considered to be the most immunostimulatory component of the whole organ allografts. Whether this property of vascular endothelium is applicable to the endothelium of dynamic valves and can be markedly altered by cryopreservation remains to be determined.

First, several studies have reported on the viability and function of donor endothelial cells of cryopreserved allograft valves (6, 50-53). Yankah *et al*. described 70% to 80% endothelial cell viability in cryopreserved human allograft valves (54, 55). However, Lupinetti *et al*. demonstrated that viable endothelial cells were only observed on 21 of 131 (16%) cryopreserved allograft specimens (51).

Most previously published studies examining the cellular viability of allograft valves used specimens obtained immediately after harvest, disinfection, or thawing, but not after subsequent implantation. Thus, influences of implantation, including immunologic consequences, were not considered. Such studies may not be able to predict long-term cellular viability in implanted valves (44, 50, 51). Previous studies demonstrated that early endothelial viability was well maintained in fresh grafts, but completely abolished in cryopreserved grafts. A previous study showed a sharp divergence between the pathologic fate of endothelium and that of fibroblast cells of cryopreserved valves and emphasized the importance of analyzing the cell populations independently. Our previous study (44) revealed the cytosolic esterase activity of cryopreserved human umbilical vein endothelial cells fell to $28\% \pm 9.0\%$ of fresh specimens, and mitochondrial dehydrogenase activity fell to $44\% \pm 6.1\%$. These findings suggest that cryopreservation appeared to produce serious damage to cytosolic and mitochondrial functions of endothelial cells. We presumed that the cell membrane is easily damaged soon after harvest. Mitochondria are the center of the intracellular energy source. The more the mitochondrial function is aggravated due to cryopreservation process, the more the cell membrane deteriorates due to energy depletion.

Loss of the endothelium may contribute to enhanced graft longevity by reducing the host immune response, which may contribute to valve degeneration. However, loss of the endothelium may increase the capacity for thrombus formation and adversely affect the underlying fibroblasts, thereby accelerating graft deterioration. It is unknown to what extent valve degeneration is attributed to immune responses.

To elucidate the morphology, mechanisms of deterioration, cellular viability, extracellular matrix integrity, and the role of immune responses in the dysfunction of cryopreserved allograft valves, Mitchell *et al*. evaluated explanted cryopreserved allograft valves and compared them with aortic valves removed from short-term and long-term transplanted hearts (18). Cryopreserved allograft valves are morphologically nonviable ; their collagen is flattened but largely preserved (18). They are unlikely to grow, remodel, or exhibit active metabolic functions, and their usual degeneration cannot be attributed to immunologic responses. In contrast aortic valves of transplanted hearts maintain near-normal overall architecture and cellularity and do not show apparent immunologic injury, even in the setting of fatal myocardial parenchymal rejection or graft arteriosclerosis. Allografts implanted for more than one day showed progressive collagen hyalinization and loss of normal structural complexity and cellularity including endothelium and deep connective tissue cells. Inflammatory cells were generally minimal or absent in the allografts. Cryopreserved allograft valves have minimal viable cells, but largely retain the original collagen network ; preservation of autolysis-resistant collagenous skeleton probably provides the structural basis of function. Although, in some studies, short-course immunosuppression

has been recommended to prevent early failure of allograft valves (56), in another study, the usual degeneration was not derived from immunological responses. These findings suggest that the immunogenicity of cryopreserved allograft valves can be markedly altered and disappears by cryopreservation process.

However, Mohan *et al.* insisted that after "better" cryopreservation, the endothelial cell function is similar to that of normal endothelium and that "a lack of viable endothelium" suggests that "preservation of the remaining valve may be the suspect" (22). Cryopreservation must aim to preserve a viable endothelial lining, because its loss could be the main reason behind the accelerated thrombosis, calcification, and infection of commercial bioprosthetic valves. Unfortunately, there is currently no clinical information to support this position. Using the occurrence of "accelerated thrombosis, calcification, and infection of commercial bioprosthetic valves", that is without endothelial lining as justification for preserving the endothelium of allografts is not sufficient.

It cannot yet be determined whether rare endothelial cells or a viable endothelial lining has a positive, negative, or neutral effect on the long-term structure and function of cryopreserved allograft aortic and pulmonary valves, respectively.

## Collagen synthesis and collagenolysis

For the long-term performance of cryopreserved allograft valves, a more important factor may be preserving the intactness of the original collagen network (36) that provides the structural basis of the valve matrix (Fig. 3). Despite the widespread use of cryopreserved valves, the balance of collagen synthesis and collagenolysis ability of the valves has not yet been clearly revealed (57-59). Controlled collagenolysis ability was suggested to play an important role in the first step of the regeneration process (18), and consequent collagen synthesis is a second step.

Lupinetti *et al.* (41, 42) demonstrated the presence of procollagen and the $\alpha_1$-(I) procollagen gene expression in allograft aortic valves after implantation for 3 days, which suggested donor fibroblast viability. Fibroblasts have been known to exist within the matrix (40) and to participate in the valve remodeling through collagen metabolism. In our previous study, the collagen content in the cryopreserved cusps was kept at the same degree as that of the fresh cusps, and the collagen synthesis ability in the cryopreserved cusps was also relatively preserved (43). However, the protein synthesis ability, including collagen synthesis, in the cryopreserved cusps decreased significantly (43). Although the collagen synthesis ability was preserved, like the findings of Lupinetti *et al.* (41, 42), such a marked reduction in protein synthesis ability may have had a significant impact on the long-term cellular viability.

The matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes that are able to digest a wide range of extracellular matrix proteins (60-62). The expression of the MMPs from the endothelial cells (63, 64), the fibroblast cells (65, 66), and ischemic myocardium (67, 68) was consecutively revealed (63, 64). MMP-1, an interstitial collagenase, is mainly produced from fibroblasts, and is known to degrade structural type I collagen in the matrix. MMP-2 and MMP-9 are mainly produced from the endothelium, and are known to be involved in the degradation of type IV collagen, which is a major component of the subendothelial basement membrane (Fig. 4).

Our previous study demonstrated that the cytosolic

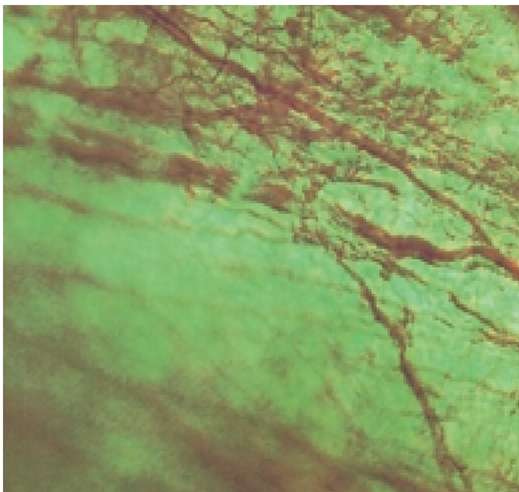

**Fig. 3. Photomicrograph of a section from a cryopreserved heart valve. Cusp of the porcine heart valve is stained with Sirius red (Muto Pure Chemicals, Tokyo, Japan), which is a dye selective to collagen. Collagen fibers stained red. (Original magnification×200).**

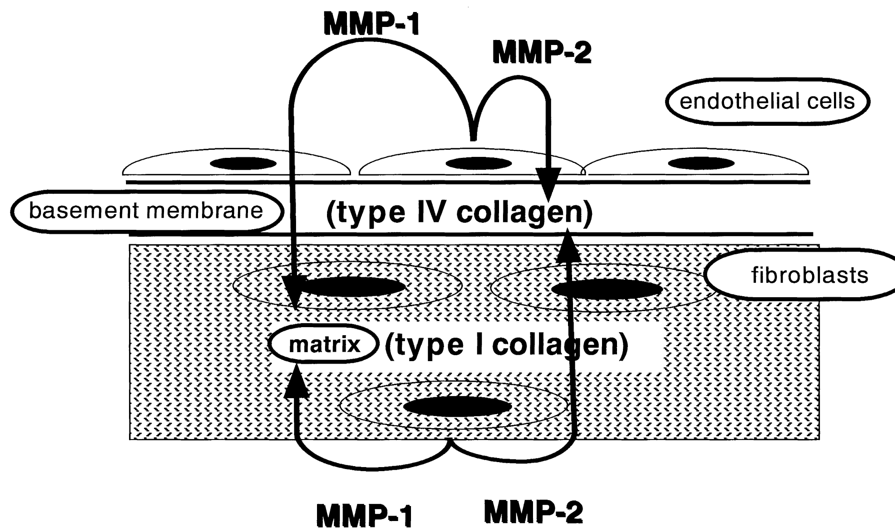


**Fig. 4. Action of the MMPs in heart valve.**
**Cellular components of heart valve consist of fibroblasts and endothelial cells. Between both, there is a basement membrane, which is known to consist of type IV collagen. Most of the matrix of the heart valve consists of type I collagen fibers. The endothelial cells and the fibroblasts have a potential to secret collagenase, MMPs. This schema shows the site of the action of MMP-1 and MMP-2. MMP-1 mainly participates in the degradation of type I collagen and MMP-2 degrades type IV collagen.**

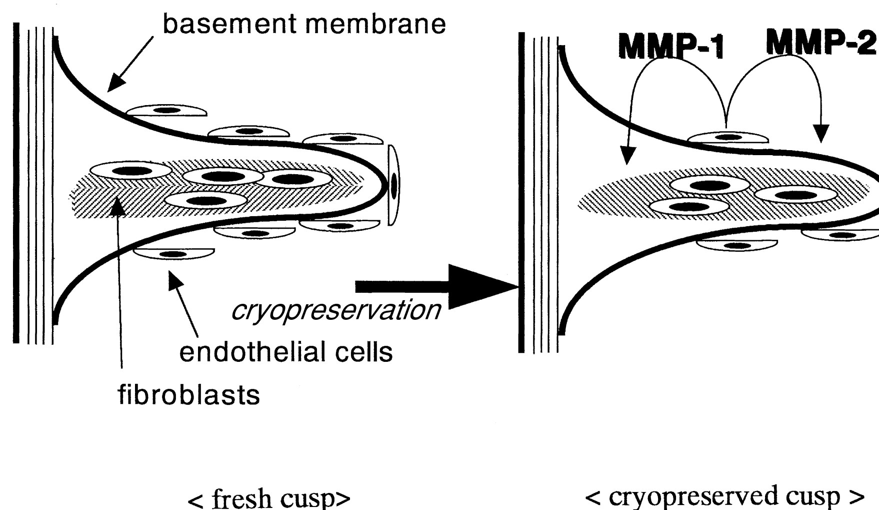


**Fig. 5. Hypothetical role of MMPs in the cryopreserved heart valve.**
**In the cryopreserved heart valve, the cryopreservation processes significantly degrade the endothelial cells covered on the basement membrane. It seems that exposed basement membrane is easily targeted by the MMPs secreted from the damaged endothelial cells.**

and mitochondrial function of endothelial cells were seriously damaged during the cryopreservation and thawing processes (44), and those processes may cause a latent cytosolic and mitochondrial injury even in the fibroblasts. Despite the anticipated reduced cellular viability, particularly endothelial cells, in the cryopreserved cusps, the activity of the MMP-1 and MMP-2 in the supernatant of the cryopreserved cusps was observed at the quantitatively same degree as those of the fresh cusps (43). These findings imply that the cryopreservation and thawing process causes MMP-1 and MMP-2 release from endothelial and fibroblast cells of allograft cusps, and activates them before the implantation (Fig. 5). There is a possibility that the activated MMP-2 will degrade the basement membrane consisting of type IV collagen and activated MMP-1 will destruct the cusp matrix. Most cryopreserved valves may have the potential to degrade and autolyze the matrix extensively during processing, intraoperative preparation, and possibly after implantation.

From these apparently discrepant results, it is suggested that the collagen synthesis ability is preserved even in the slightly injured fibroblast cells of cryopreserved valves, however, the protein synthesis ability might be extremely reduced because of limited endothelial

collaboration (43, 69, 70) and the decrease in the protein production relevant to the intracellular organellar function (44), which maintains the cell viability, and that the collagenolytic activation occurs just before implantation (43). With respect to those findings, the injured fibroblasts are going to be non-viable, collagen synthesis is suggested to deteriorate, and the original collagenous skeleton degrades in the near future. The collagenolysis dominant state was suspected in the cryopreserved valves.

Although it is necessary to elucidate the dynamics of tissue inhibitors of MMPs in the cryopreserved valves, the control of the MMPs activity in the cryopreserved valves may become more effective to obtain the long-term durability rather than the preservation of collagen synthesis ability. Recently, there were some reports that doxycycline, a derivative of tetracycline, suppresses the development of the elastase induced abdominal aortic aneurysms (71, 72), through the direct MMPs-inhibiting activities. Other MMPs inhibitors, marimastat (BB-2516), batimastat (BB-94) and BE16627B, were also developed as agents for inhibiting cancer metastasis (73-75). These agents might improve the allograft preservation in clinical use.

In conclusion, the findings of this review suggest the possibility that the current cryopreservation techniques and management, themselves, may lead a cryopreserved valve to become non-viable acellular tissue by the collagenolytic activation and degradation of protein synthesis, which will cause the destruction of the valve matrix. Further examination of collagen metabolism and modulation of the collagenolytic activity will be necessary to improve the allograft preservation for better clinical use. We believe that the enthusiasm for allograft tissue implantation should be considered in the overall context of allograft failure.

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