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Factors Affecting Cellular Viability During Preimplantation Processing

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Extensive clinical durability of allograft valves has long been suggested to be linked to cellular viability and extracellular matrix integrity at the time of implantation.¹⁻³ Efforts to standardize processing procedures for valve transplantation and optimize the longevity of the valves provided the original impetus for researchers to examine the effects of each processing step. This chapter focuses on the series of studies that resulted from this work. As reviewed in the previous section, viability can be evaluated in a number of ways, depending on which parameters are of interest for the study. In this chapter, we summarize the results using various methodologies to assess the health of leaflet cells following preimplantation processing.

Porcine Cardiac Valve Leaflet Studies

Initial studies noted that clinical harvesting of allograft heart valves necessitates a time period of warm ischemia, corresponding to the time from cessation of donor heartbeat to the time of transport. Leaflet cell metabolic response to varying warm ischemic time intervals was characterized in porcine aortic valve leaflets by magnetic resonance spectroscopy (Figure 18.1).⁴ Two hours following donor death, aerobic metabolism ceased, as evidenced by total depletion of adenosine triphosphate (ATP), no phosphorus production, and signifi-

cant lactate accumulation; these results suggest that oxidative phosphorylation stopped, high energy phosphate stores were depleted, and metabolism was converted to anaerobic processes. At 24 hours, proline incorporation ended, signifying that protein synthesis had ceased. Between 24 and 36 hours after death, lactate production ceased, showing that anaerobic metabolism had stopped, and most cells exhibited ultrastructural evidence of irreversible cellular injury.

Initial ischemia may be only one factor in the series of valve preparation steps that leads cumulatively to interstitial cell injury. After variable ischemic times, cardiac valves undergo two additional steps prior to transplantation: antibiotic disinfection and cryopreservation. Early metabolic markers of cellular injury were assessed biochemically via high performance liquid chromatography (HPLC) analysis of components of the adenine nucleotide pool.⁵ This cascade of high-energy phosphates and purine byproducts is a dynamic metabolic pathway with elements that react quickly to metabolic changes and respond with substrate deprivation or enzymatic inhibition. The aims of this study were to define the reduction in energy reserves of leaflet cells during the initial phases of processing-associated injury, and to determine if such damage might be reversible. These changes in phosphate metabolism may ultimately lead to changes in energy-dependent cell functions including proliferation, protein transport, and synthesis (Figure 18.2). While variable periods of ischemia were examined,

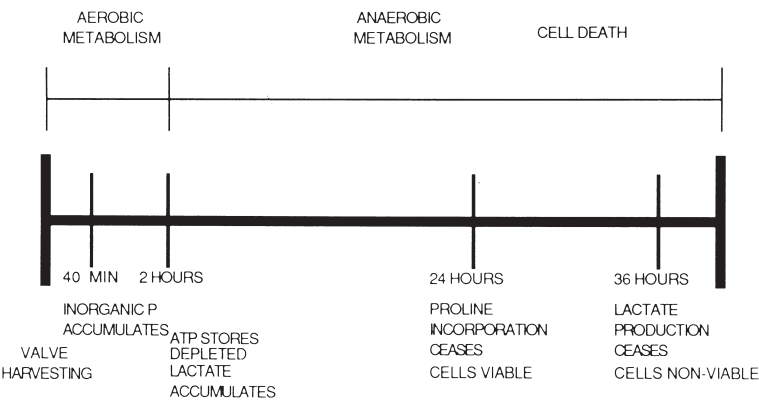


FIGURE 18.1. Progression of porcine aortic valve leaflet metabolic state in response to increasing ischemic time. Phosphorus accumulates significantly following 40 minutes post harvest warm ischemia. Metabolism becomes anaerobic after two hours, as shown by depletion of ATP reserves and accumulation of lactate. Viable cells persist with anaerobic metabolism, up to 24–36 hours.

the maximal 24-hour period of harvest ischemia did not fully deplete ATP or total adenine nucleotides (TAN). Subsequent pre-implantation processing steps of disinfection and cryopreservation decreased ATP and TAN following any ischemic interval, with the greatest damage following the longest ischemic time of 24 hours. However, even under the most stressful conditions, leaflet cells maintain significant metabolic capacity, as evidenced by residual high energy phosphate reserves.

A subsequent series of experiments was performed to separate out further the individual effects of the warm ischemic time, cold ischemic time, antibiotic treatment, and cryopreservation steps on cells' metabolic states (Table 18.1) (Figure 18.3).⁶ As demonstrated in the previous study, warm ischemic times up to 24 hours did not appear to have significant detrimental effects on porcine aortic valve leaflet cells. Following an additional 24 hour cold ischemic time, there was a 17% reduction in ATP, and

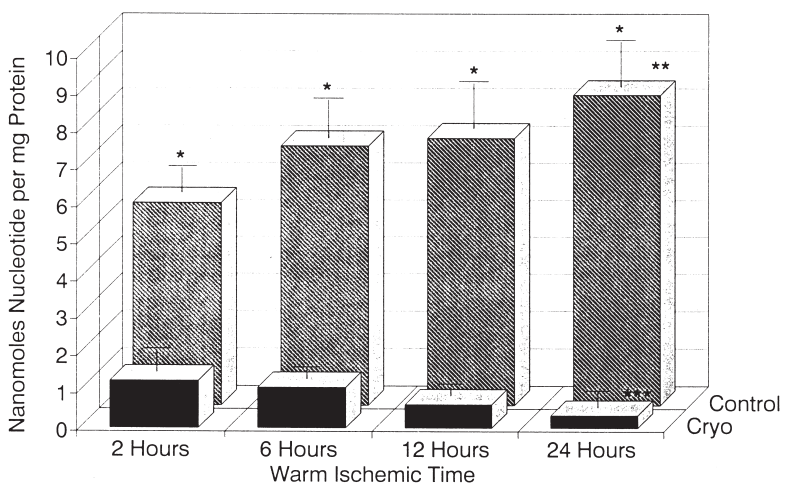


FIGURE 18.2. Total adenine nucleotides ([TAN] = [ATP] + [ADP] + [AMP]) of control leaflets (exposed to cadaveric ischemia only) and cryopreserved leaflets (cadaveric ischemia and cryopreservation processing). At each ischemic time, antibiotic treatment and cryopreservation lead to dramatic reduction in TAN. TAN of control leaflets are significantly higher after 24 hours; this accumulation may

reflect ATP degradation or partial metabolic restoration through other salvage pathways. Leaflets undergoing 24hrs ischemia and cryopreservation exhibit the largest reductions. (*P < 0.05 control versus corresponding ischemic time cryopreservation groups; **P < 0.05 control versus 2 hr controls; ***P < 0.05 in 24hr cryopreserved versus 2 and 6hr cryopreserved groups). CRYO, cryopreserved.

TABLE 18.1. Levels of Metabolites (nmol/mg Protein)^a Following Each Leaflet Processing Phase.

	Group I (WIT only)	Group II (WIT + 24 h 4°C Ischemia)	Group III (WIT + 24 h 4°C Antibiotic Disinfection)	Group IV (WIT + 4°C Ischemia + Cryopreservation)	Group V (WIT + 4°C Disinfection + Cryopreservation)
ATP	1.78 ± 0.25	1.48 ± 0.12	0.91 ± 0.13 ^b	0.46 ± 0.11 ^b	0.25 ± 0.04 ^b
ADP	0.59 ± 0.07	0.55 ± 0.05	0.33 ± 0.07	0.27 ± 0.05	0.25 ± 0.03
AMP	0.40 ± 0.10	0.78 ± 0.21	1.00 ± 0.40	0.14 ± 0.07	0.18 ± 0.07
Adenosine	0.18 ± 0.06	1.20 ± 0.29	6.10 ± 3.50	0.52 ± 0.10	3.10 ± 0.70
Inosine	1.50 ± 0.30	0.72 ± 0.20	0.87 ± 0.20	0.50 ± 0.50	0.71 ± 0.13
Hypoxanthine	1.70 ± 0.25	0.78 ± 0.16	0.73 ± 0.24	0.12 ± 0.06	0.07 ± 0.03
Xanthine	0.00 ± 0.00	1.10 ± 0.63	0.10 ± 0.08	0.09 ± 0.08	0.05 ± 0.04
NAD ⁺	0.50 ± 0.04	0.33 ± 0.04	0.29 ± 0.12	0.07 ± 0.04	0.23 ± 0.04

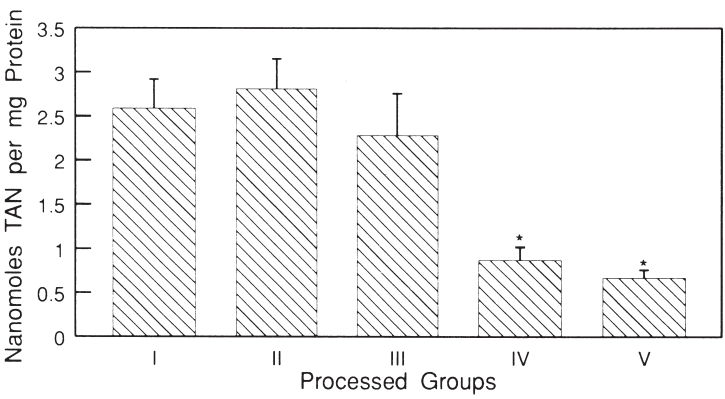
^a Adenine nucleotide metabolite concentrations (± standard error of the mean) existing at the completion of each step of preimplantation processing. ^b $p < 0.05$ vs, group I by analysis of variance. ADP = adenosine diphosphate; AMP = adenosine monophosphate; ATP = adenosine triphosphate; NAD⁺ = nicotinamide adenine dinucleotide, oxidized form; WIT = warm ischemia.

evidence that ATP degraded only to ADP and AMP, perhaps because ATP was not broken down completely or was partly restored through other pathways. Cold antibiotic disinfection reduced the leaflets' ATP by almost 50%, while increasing purine byproducts 55%; thus, half of the ATP was broken down to ADP and AMP, and the TAN partly degraded to adenosine, inosine, hypoxanthine, and xanthine. These results suggest that most of the degraded ATP was broken down in a reversible way; this is important since maintaining total high energy phosphates (not just ATP) is crucial to a cell's basal functional capacity. Of all the processing steps, however, cryopreservation is responsible for the largest reduction in ATP (74%). The consequences of the cryopreservation step

augment the effects of cold ischemic and antibiotic disinfection. Therefore, in the effort to balance beneficial processing steps with detrimental ones, antibiotic treatment appears to be a useful step; it does not have a high relative metabolic cost in the overall cryopreservation process. All steps in the processing were synergistic in their reduction of the adenine nucleotide pool. However, even after the complete harvesting and cryopreservation processing, some leaflet cells maintained quantifiable high-energy phosphates. This correlated with the morphologic studies in suggesting that some cells were able to tolerate processing-related stresses.

Because leaflet cells appeared to retain some metabolic capacity following processing, treat-

FIGURE 18.3. Total adenine nucleotides ([TAN] = [ATP] + [ADP] + [AMP]) measured after each step in leaflet processing; (I) warm ischemia, (II) cold transport, (III) antibiotic disinfection, (IV) cryopreservation, and (V) cryopreservation with antibiotics. (* $P < 0.05$ versus groups I, II, III.)



ment with inhibitors of adenosine deaminase and nucleoside transport was explored in an attempt to salvage adenosine pools and as validation of the degradation observations (Figure 18.4 and 18.5).⁷ HPLC showed that with minimal ischemic times (40 minutes) the processing steps of disinfection and cryopreservation independently disrupt the ATP-ADP cycle. However, treatment with restitution therapy maintained nucleotide levels at baseline harvest concentrations, and so such agents may be able to protect leaflet cells from catabolism, as is possible in myocardium. This study provided

further evidence that although injured, the entire valve leaflet cell population does not become metabolically inert following ischemia and processing, and in fact, cumulative metabolic damage may be minimized via adenine nucleotide protection.

The HPLC series of experiments served to demonstrate profound energy store depletion yet some potential for manipulation of the metabolic pathways. Without such protection and in fact by using the harvesting and processing protocols contemporary in the 1980s and 1990s, these experiments suggested that

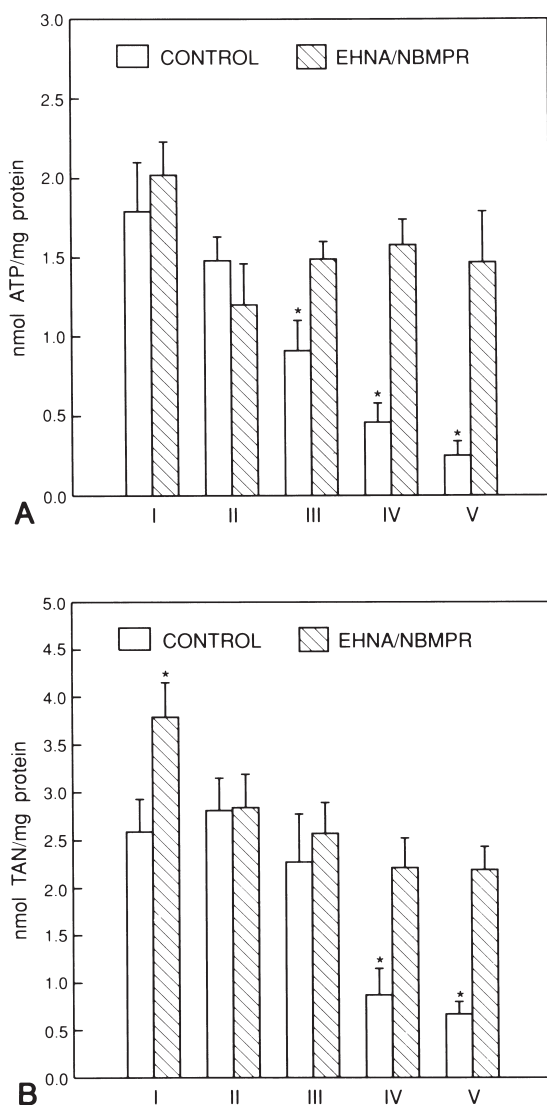


FIGURE 18.4. Adenine nucleotide levels in control valves and in valves treated with restitution therapy consisting of the nucleoside transport inhibitor p-nitrobenzyl-thioinosine (NBMPR) and the adenosine deaminase inhibitor erythro-9 (2 hydroxy—3-nonyl) adenine (EHNA). (A) ATP levels. Significant reductions are seen following disinfection (control group III), cryopreservation after warm and cold ischemia only (control group IV), and cryopreservation after warm ischemia and cold disinfection (control group V). EHNA/NBMPR-treated groups showed prevention of ATP loss (* $p < 0.05$ versus control group I and corresponding EHNA/NBMPR-treated group). (B) Total adenine nucleotide levels ($[TAN] = [ATP] + [ADP] + [AMP]$). Disinfection processing (group III) did not result in significant depletion of TAN as it did with ATP. This likely reflects an early phase of processing-associated damage with respect to phosphorylated adenine nucleotides. Processing steps in groups IV and V did lead to significant losses in TAN, which were prevented by EHNA/NBMPR treatment (* $p < 0.05$ versus control group I).

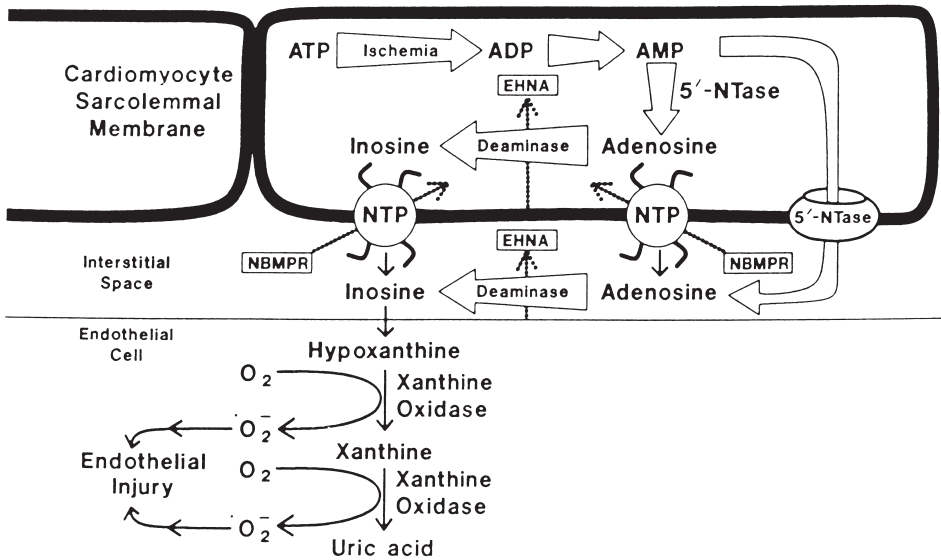


FIGURE 18.5. Diagram of biochemical pathways and active sites of EHNA/NBMPR. Nucleoside transport protein (NTP) facilitates adenosine and inosine export. NBMPR allows intracellular retention of adenosine. EHNA competes with adenosine deami-

nase to inhibit it both within the cell and extracellularly. Adenosine hydrolysis to adenosine is almost completely prevented, leading to "restitution": increases in phosphorylated adenine nucleotides by reversal of the degradation pathway.

while many cells might be morphologically intact at the time of implantation, they were likely "doomed" to early death simply from metabolic depletion.

Human Cryopreserved Cardiac Valve Homografts

To determine if the response of human leaflet cells to clinical pre-implantation processing was similar to that observed in porcine valves, a series of human valves was subjected to the following: a warm ischemic interval in the donor of between 0 and 20 hours, followed by 24 hours of cold antibiotic disinfection, then cryopreservation. It is important to note that each human valve was originally intended for transplantation, and was included in the study only when it was deemed unsuitable, typically for anatomical reasons. Because of its initial designation, each valve in the study underwent the complete series of cryopreservation processing steps, in contrast to the processing in much of the com-

parative work on porcine valves. The effects of these processing steps on cell metabolic state and ultrastructure were assessed with HPLC and electron microscopy (Figure 18.6).^{8,9} Warm ischemic intervals of less than 2 hours resulted in virtually no evidence of damage at the ultrastructural level. Generally, the longer the ischemic time interval, the greater the extent of cellular injury. Ultrastructural changes providing evidence of damage gradually increased over time from endoplasmic reticulum dilatation, cytoplasmic edema, and mitochondrial swelling, to mitochondrial flocculent densities, karyolysis, and plasma membrane disruption. Up to 12 hours of warm ischemia, cells exhibited morphology reflecting mostly reversible cellular injury, with minimal ultrastructural evidence of irreversible injury. However, after 12 hours, evidence of irreversible damage increased dramatically, and the amount of injury correlated with increased warm ischemic time. Following 20 hours of warm ischemia, 80% of the cells were injured. In contrast, in porcine valves subjected to warm ischemia but no other processing, ultrastructural evidence of irreversible

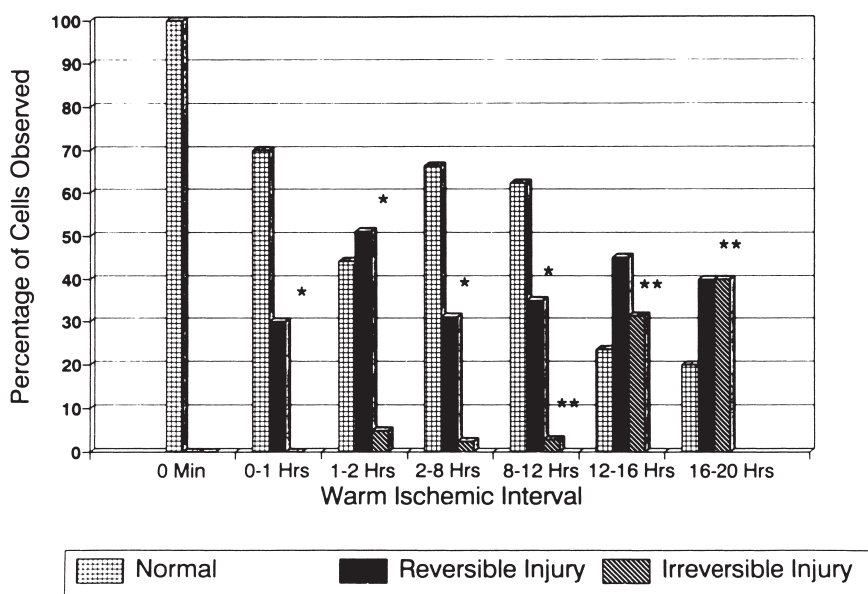


FIGURE 18.6. Percentage of human aortic valve leaflet cells exhibiting normal ultrastructure of evidence of reversible or irreversible cellular injury. Time intervals of warm ischemia for each group are noted on the x-axis. Cochran-Mantel-Haenszel

trend analysis demonstrates a positive correlation (* $p < 0.0001$) for reversible injury during the first 12 hours of warm ischemic time and a positive correlation (* $p < 0.001$) between 12 and 20 hours of warm ischemia for irreversible cellular injury.

cellular injury manifested later than in this study's human valves—detectable only after 24 hours of warm ischemia. Taken together, these studies suggest that post-ischemia processing steps contribute substantially to cellular damage in human cryopreserved homografts.

The total adenine nucleotide depletion was substantial at harvest ischemic times greater than 2 hours (Table 18.2; Figure 18.7).⁹ ATP depletion was similar to that observed in porcine experiments, and in 76% of the valves, ATP, ADP, and AMP were undetectable. Increased levels of catabolites confirmed that energy consumption was extremely high, and depletion occurred predominantly during pre-implantation processing rather than during harvest associated ischemic time.

Another assay of membrane integrity and metabolic activity, measurement of 3H-2deoxyglucose phosphorylation, has been used to evaluate human leaflets after cryopreservation.¹⁰ Cryopreservation processing steps as described above resulted in a 30% decrease in phosphorylation as compared to fresh

valves. This decrease could be partially reversed by incubating the valves at 37°C prior to transplantation.

These experiments supported the concept of a “stunned” leaflet cell population following processing, a population of cells devoid of energy reserves and thus at high risk for cell death, during the stress of transition following implantation. These findings possibly explained the potentially conflicting data that purported to show excellent cell “viability” as defined by morphology but contrasted with sparse cellularity at leaflet explants.^{2,11}

Resuscitation of Leaflet Cells

Taken together, the work of other researchers and our own studies of porcine and human valves provided evidence that pre-implantation processing had significant detrimental effects on leaflet interstitial cell viability and functionality. The work also demonstrated consistently that a few robust cells remain following

TABLE 18.2. Metabolic Concentrations of the Adenine Nucleotide Pool (Individually Eluted, nmol Metabolite/ing Leaflet Protein) in Cryopreserved Human Valves.

Valve	WIT	Type	Donor Age/Sex	Cause of Donor Death	Reason Rejected	ATP	ADP	AMP	ADO	INO	HX	X	Urate	NAD ⁺
1	<1	Aortic	5/M	Suffocation	+Cx	0	0	0	1.870	0	0	0	0	0
2	<1	Aortic	46/F	Multiple trauma	+Cx	0.355	0.266	0	1.060	0.887	0	0	0	0.266
3	<1	Aortic	9/M	Multiple trauma	+Cx	0	0.442	0.737	2.500	1.470	0	0	0	0
4	1	Aortic	49/F	Multiple trauma	+Cx	0.404	0.101	0.908	1.410	1.110	0	0	0	0.303
5	1	Pulmonic	33/F	Multiple trauma	TE	0	0.327	0.762	0.544	0.436	0	0	0	0
6	1	Aortic	11/F	Multiple trauma	TE	0.663	0.663	1.330	0.663	0.442	0	0	0	0.221
7	3	Pulmonic	18/M	Multiple trauma	Ann.hem.	0	0	0	2.220	0.910	0	0	0	0
8	4	Pulmonic	2/M	Multiple trauma	TE	0	0	0	1.240	0.742	0	0	0	0
9	4	Aortic	14/M	Multiple trauma	+Cx	0	0	0	0.271	1.080	0.452	0	0	0.181
10	4	Aortic	44/F	Multiple trauma	TE	0	0	0	0	2.280	0	0	0	0
11	6	Aortic	8/M	CHI/MVA	SD	0	0	0	0	0	0	0	0	0
12	6	Aortic	3/F	CHI/MVA	TE	0	0	0	2.072	0.777	0	0	0	0.259
13	7	Aortic	40/F	MI	+Cx	0.351	0	0	1.520	0	0	0	0	0.234
14	8	Pulmonic	40/F	MI	+Cx	0	0	0	0.940	0.416	0	0	0	0
15	9	Aortic	33/F	MI	+Cx	0	0	0	0.584	0.973	0	0	0	0.195
16	9	Pulmonic	33/F	MI	+Cx	0.433	0	0	0.433	0	0	0	0	0.173
17	9	Aortic	7/M	CHI/MVA	+Cx	0	0.169	0	0.674	0.590	0	0	0	0.169
18	10	Pulmonic	7/M	CHI/MVA	+Cx	0	0	0	0.420	0.737	0	0	0	0
19	11	Pulmonic	47/M	Multiple trauma	+Cx	0.357	0	0	0.990	0.713	2.420	0	0	0.143
20	13	Aortic	23/M	Multiple trauma	+Cx	0	0	0	0.500	0	0	0	0	0
21	13	Aortic	35/M	Multiple trauma	+Cx	0	0	0	0.504	0.705	0	0	0	0.201
22	15	Aortic	16/F	Multiple trauma	+Cx	0	0	0	0.294	0	0	0	0	0
23	16	Pulmonic	19/M	Multiple trauma	+Cx	0	0	0	0.813	0	0	0	0	0
24	20	Aortic	27/M	Multiple trauma	+Cx	0	0	0	0.204	0.511	0	0	0	0
25	20	Aortic	19/F	CHI/MVA	+Cx	0	0.343	0	0.257	0.429	0	0	0	0

WIT = warm ischemic time; ATP = adenosine triphosphate; ADP = adenosine diphosphate; AMP = adenosine monophosphate; ADO = adenosine; INO = inosine; HX = hypoxanthine; X = xanthine; NAD⁺ = nicotinamide adenine dinucleotide (oxidized form). Clinical characteristics of cryopreserved donor valves were assayed by HPLC. CHI = closed head injury; MVA = motor vehicle accident; CHF = congestive heart failure; MI = myocardial infarction; TE = technical error; +Cx = positive donor blood culture; Ann. Hem. = annular hematoma; age in years; M = male; F = female.

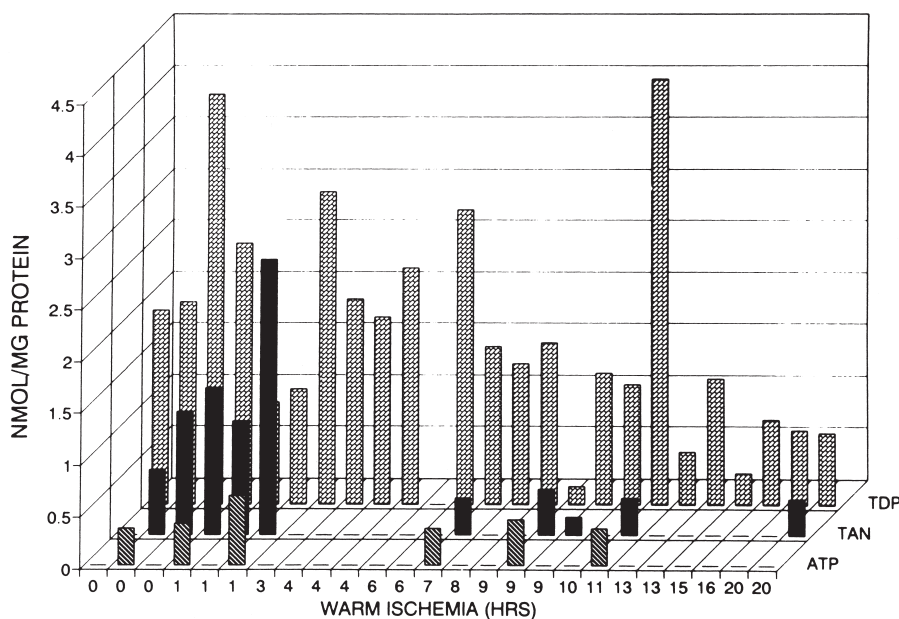


FIGURE 18.7. Depletion of adenine nucleotide pool in cryopreserved human valves. Major high energy phosphates and catabolites are only slightly retained at shorter ischemic time intervals (x-axis). This graft suggests that processing contributes more than

ischemia to leaflet energy losses. Key: Σ [ATP] + [ADP] + [AMP]; TDP, Σ [adenosine] + [inosine] + [xanthine] + [hypoxanthine]. Front bars, ATP; middle bars, TAN; rear bars, TDP.

ischemia, disinfection, and cryopreservation. Yet, the presence after processing of even a small number of live leaflet cells with metabolic reserves and intact healthy ultrastructure led investigators to hypothesize that perhaps leaflet cells could be resuscitated prior to implantation (for review see Chapter 14). In fact, incubation of cryopreserved porcine aortic valves in organ culture (37°C, media with 15% serum) for 8 days prior to implantation was able to restore the normal LIC population and matrix composition to the leaflets (Figure 18.8).¹²

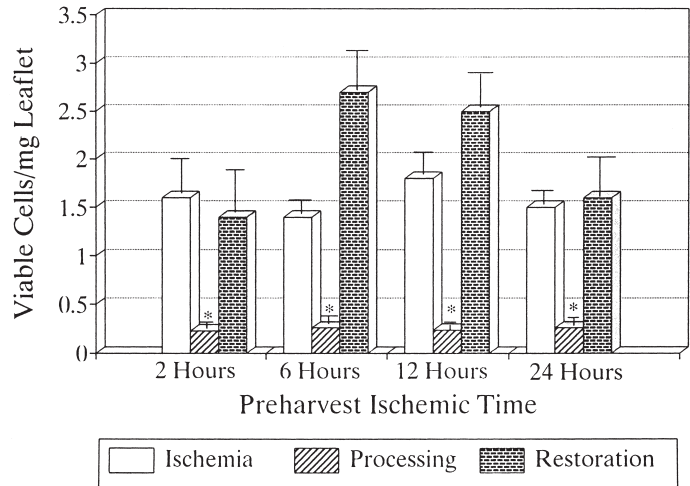
Ovine Implant Model

The preceding body of work has provided a careful analysis of the effects of pre-implantation processing on various aspects of leaflet viability, and furthered our understanding of the state of the leaflet at the time of implantation. Of ultimate importance is the state of the valves, especially the state of the cells

and matrix, while in the recipient. Another recent study has examined leaflet cellularity after implantation into a chronic ovine model, with a specific focus on the mechanisms responsible for cell loss.¹³ This study has specifically addressed the hypothesis that apoptosis may be the mechanism underlying the acellularity seen in implanted aortic allograft valves. In an ovine model, fresh and cryopreserved aortic valves were examined following implantation times ranging between 2 days and 20 weeks. Leaflet interstitial cells exhibited losses in proliferating cell nuclear antigen (a marker of mitotic function) as well as positive nick end labeling, nuclear condensation, pyknosis, and formation of apoptotic bodies containing remnants of nuclear material. This evidence of mitotic cessation and apoptosis was detectable by 2 days following implantation. It reached a peak at 10–14 days, and by 20 weeks, grafts were completely acellular.

The cell state of human cryopreserved allograft valves and valves of transplanted hearts

FIGURE 18.8. Viable leaflet interstitial cells. Cells were released from leaflets by collagenase digestion, and viability was assessed via Trypan blue exclusion. Groups: Ischemia – warm ischemia only. Processing – ischemia + disinfection + cryopreservation. Restoration – processing + organ culture, M199 15% FBS, 37°C, 8 days. Processing significantly reduced cell numbers, but restoration treatment led to dramatic repopulation of the leaflets.



that did not undergo cryopreservation have recently been compared by Mitchell et al., 1998.¹⁴ Explanted allograft valves displayed decreased cellularity, with morphological similarities to the valves of this study. In distinct contrast, aortic valves of orthotopically transplanted hearts maintained normal cellularity and morphology. Pre-implantation processing of allograft valves differs from that for heart allografts in that it contains periods of hypoxia, disinfection, and cryopreservation; this work gives further support to the idea that valve processing contributes to the loss of leaflet cellularity and the development of apoptosis, as well as as possibly implicating an immune component.

Conclusion

The metabolic and morphological studies from the our laboratory have defined a severely stressed population of donor cells. Such metabolic “stunning” is by itself likely inconsistent with survival for a large fraction of the cell population unless some intervention is made prior, during, or after transplantation. The apoptosis findings suggest that this stress could result in limited long-term viability by two mechanisms. First, the early phase cell survival is likely low following transplantation due to the limited cellular energy reserves. Second, either the stress of pre-implantation processing or the abnormal environment in which the injured matrix cells

find themselves following transplantation (in particular, no native endothelium) triggers the apoptosis sequence. This could fit with some observations of moderate matrix and cellular viability early after implantation. Means by which to promote and augment valve viability in the recipient remain to be fully developed, and successful initial efforts at resuscitation through organ culture lend an optimistic note to these efforts. However, mere retention of donor cells may allow for preservation of synthetic function for a time following transplantation. These retained cells could restore matrix components and thus initially enhance physical leaflet characteristics and by this actually improve durability. This potential value of retained donor cells has to be balanced against the negative effects of provoking an immune response (*vida infra*). Cell loss by apoptosis may be preferable to necrosis as the former process is by definition non-inflammatory while necrotic cell debris are both extremely inflammatory and antigenic and could lead to accelerated degeneration, fibrosis and calcification of the transplanted allograft valve tissues.

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