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Viability and Enzymatic Activity of Cryopreserved Porcine Heart Valve

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Abstract

Fibroblast viability of a natural tissue valve for replacing a defective heart valve through allograft or xenograft has been suggested to affect its clinical durability. In this study, the cell viability and enzymatic activity of porcine heart valve leaflets were examined in regard to concerning to the preservation process [variable warm ischemic time (WIT), cold ischemic time (CIT), and cryopreservation]. Porcine heart enblocs were obtained and valve dissection was performed after 2, 12, 24, or 36 hours, in respective groups A, B, C, and D, as WIT. Each group was stored for 24 hours as CIT and cryopreserved. Leaflets were dissected from a valved conduit after each process, and cell viability and enzymatic activity in the leaflet were investigated using trypan blue staining and API ZYM kits. WIT extension significantly decreased fibroblast viability ($p<0.05$, $92.25\pm2.7\%$ at 2 hours, $84.9\pm6.7\%$ at 12 hours, $57.0\pm10.2\%$ at 24 hours, $55.9\pm7.9\%$ at 36 hours), while CIT for 24 hours was also influenced significantly ($p<0.05$), whereas cryopreservation demonstrated no effect on cellular viability. In enzyme activity observation, several enzymes related to lipid or nucleotide degradation (esterase, esterase lipase, particularly phosphatase, phosphohydrolase) were remarkably changed following the valve-fabrication process. After 24 hours CIT, these enzymatic activities in groups B, C and D significantly increased, but the activities decreased after cryopreservation. Particularly, both the viability and enzymatic activity showed remarkable changes after CIT in group B (WIT=12 hours). These results suggest that WIT is more important than CIT in maintaining viability of the valve, and that completing all the cryopreservation process within 12 hours after acquisition is recommended.

Key Words: Porcine valve, viability, enzymatic activity, tissue preservation, cryopreservation

INTRODUCTION

The use of metallic valves or bioprosthetic tissue valves often produces serious clinical problems. Thrombogenesis and the consequent clinical side-effects can occur on metallic valves.[1] In comparison, a tissue valve for homograft or xenograft has several advantages such as freedom from coagulation, low incidence of thromboembolism, good physiologic performance, and relative resistance to endocarditis,[2-4] however it has demonstrated less durability than a mechanical valve.[5]

To increase the durability of a tissue valve, glutaladehyde has generally been used as a chemical cross-linker for collagen to promote the stability of collagenous extracellualr matrix of the tissue. But it has also been reported that a chemically-treated valve reveals less durability than homograft after implantation because of toxicity or calcification derived from the chemical reagent.[6,7] By comparison, cryopreserved homograft has shown favorable clinical results after implantation, and the reason seems to be attributable to the viable fibroblasts in the valve.[8,9] Viable fibroblasts synthesize the main constituents of extracellular matrix: collagen, elastin, reticulin and mucopolysaccharides;[10-13] and therefore, the viability of fibroblasts in the implanted valve is related to the longevity of the implantation.

The presevation method of an acquired tissue influences the viability of fibroblast. Harvesting, the interval between arrest of the donor's heart and preservation of valves in refrigeration (the so-called warm ischemic time), sterilization, cryopreservation and thawing are the primary factors which affect the cellular physiology.[14-16] All harvested valves have an obligatory period of ischemic time. The ischemic time is divided into warm ischemic time (WIT), which is

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defined as the interval from heart cessation until procurement of the heart, and cold ischemic time (CIT), which is the interval between procurement and dissection.[17-18] This ischemic time has been shown to affect cell viability, but the time-dependent damage response of tissue valves has not been well characterized.

In this study, we investigated WIT-dependent damage to porcine leaflet tissue by investigating the fibroblast's viability and enzyme activities after 24 hours CIT and cryopreservation.

MATERIALS AND METHODS

Preservation of specimens

Thirty-two porcine heart-lung enblocs, consisting of blood-filled heart, lung, aorta, and vena cava, were obtained at a local slaughterhouse. The enblocs were preserved at 4−8°C in a refrigerator for 2, 12, 24, and 36 hours as WIT, and respectively assigned to groups A, B, C, and D. In each group, cellular viability and enzymatic activities were investigated after WIT, CIT and cryopreservation.

The left coronary valve leaflets were excised after each planned WIT. The remaining conduits were stored in Hartmann's solution (Choongwae Pharmacia Co. Seoul, Korea) at 4°C for 24 hours, and each non-coronary leaflet was excised to investigate the effects of 24-hours CIT. Subsequently, the last remaining conduits with the right coronary leaflets were disinfected in RPMI 1640 medium-antibiotic solution (cefoxitin 240 μg/ml medium; lincomycin 120 μg/ml; polymycin 100 μg/ml, vanocomycin 50 μg/ml) at 4°C for 24 hours. The conduits were then cryopreserved for 2 weeks through a standard cryopreservation protocol. Specimens were placed in cryopresevation-bags containing RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO), and cryopreserved in a vapor phase of liquid nitrogen in a programmed freezer (MVE Cryogenics, Minneapolis, MI, USA). After 2 weeks, the valves were placed in a water bath at 40°C, and then stepwise dilution of DMSO for thawing was done.[14,19] The thawed right coronary leaflets were used as the cryopreserved specimens.

Assessment of viability for fibroblasts

The leaflet was placed in an Eagle's minimum essential medium (Eagle's MEM, Gibco BRL, Grand Island, NY, USA) containing 0.5% type II collagenase (350 units/mg solid, Sigma, St. Louis, MO, USA) at 37°C for 5 minutes. The endothelial layer was removed from the leaflet by gentle rubbing with a cell scraper and the tissue was minced with scissors after thorough washing with phosphate buffered saline (PBS) solution. The pieces were placed again in Eagle's MEM containing 0.5% type II collagenase and incubated in a shaking incubator at 37°C for 30 minutes. The suspension was then diluted with Eagle's MEM and centrifuged at 300 rpm for 5 minutes. The consequent supernatant was centrifuged again at 1000 rpm for 5 minutes and the obtained cell pellet was separated to dilute in Eagle's MEM. Fifty μl of 0.4% trypan blue solution (Gibco BRL, Grand Island, NY, USA) was added to 50 μl of cell suspension ($2\times 10^5-10^6$/ml). The number of cells not stained by trypan blue was immediately counted by a light microscope with a hematocytometer. The viability percentage of fibroblasts was recorded by subtracting the number of stained nonviable cells from the total number of cells.[20,21]

The measurement of enzymatic activities

The enzymatic activities of 19 enzymes were observed to investigate the overall enzymatic change in tissue by using an API ZYM kit (Biomerieux Vitek Inc., Lyon, France) which detects a phosphatase alkaline, 3 lipases [esterase (C4), esterase lipase (C8), lipase (C14)], five peptidases [leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotryipsin], a phosphatase acid, a phosphohydrolase, and 8 glucidases[α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β glucosaminidase, α-mannosidase, α-fucosidase]. Leaflet tissue was ground with a potter's tube, and suspended in 2 ml of sterilized distilled water. The suspension was centrifuged at 400 rpm, and the supernatant was retained as specimen. Sixty-five μl of specimen was inoculated into an API ZYM strip composed of 20 microtubes containing enzymatic substrate at the bottom. The strip was incubated for 4 hours at 37°C, and each one-drop of ZYM A and ZYM B was added to the strip. An enzyme reaction was graded from 0 to 5 (enzymatic score) according to the intensity of the colored reaction as compared with representations on a color chart.[22] The enzymatic activity was represented by the quantity of hydrolyzed substrate that was determined by enzymatic score. To

secure an accurate comparison between specimens, the enzymatic score was calibrated by using the amount of protein in the loading specimen. The calibrated data revealed the value of enzyme activity per amount of protein (nmole/μg). Protein contents were determined by the Bradford method.[20] Enzymatic activity was not performed on group C after WIT due to specimen deterioration.

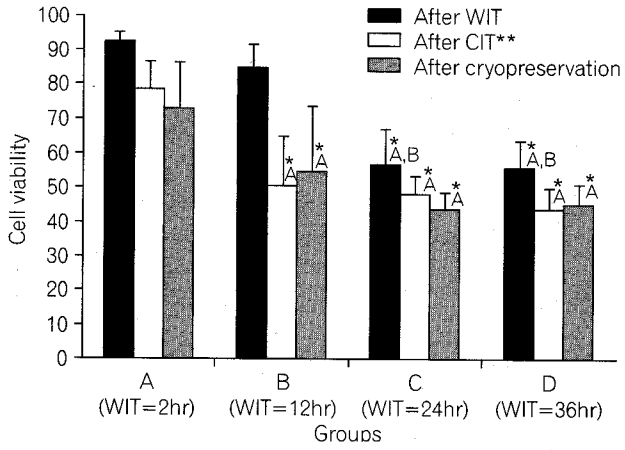

*Fig. 1. The effect of warm ischemic time, cold ischemia, and cryopreservation on fibroblast viability (%): *Groups demonstrated significantly decreased cell viability by one-way ANOVA ($p<0.05$, ^{*A}compared with group A, ^{*A,B}compared with group A and B). **Cold ischemia remarkably influenced the viability ($p<0.05$) by 2-way ANOVA, whereas cryopreservation did not ($p=0.656$).*

Statistical analysis of data

Statistical analysis was performed using the statistical package SPSS-PC. One-way analysis of variance (ANOVA) followed by Turkey HSD tests for multiple comparisons were used to detect the effect of WIT on both the viability of fibroblast and the enzymatic activity of leaflets. Pearson correlation test was performed to identify a linear association between viability and WIT. In addition, the effect of cold ischemia or cryopreservation was also determined by using two-way ANOVA. The differences of enzymatic activity between experimental processes at each group were analyzed by one-way ANOVA or by t-test. A statistically significant difference was accepted as $p<0.05$.

RESULTS

Fibroblast viability related to variable WIT, cold ischemia, and cryopreservation is shown in Fig. 1. As WIT increased from 2 hours to 36 hours, the cellular viability was reduced from 92.15±2.67% to 55.88±7.88%, and a significant negative correlation was

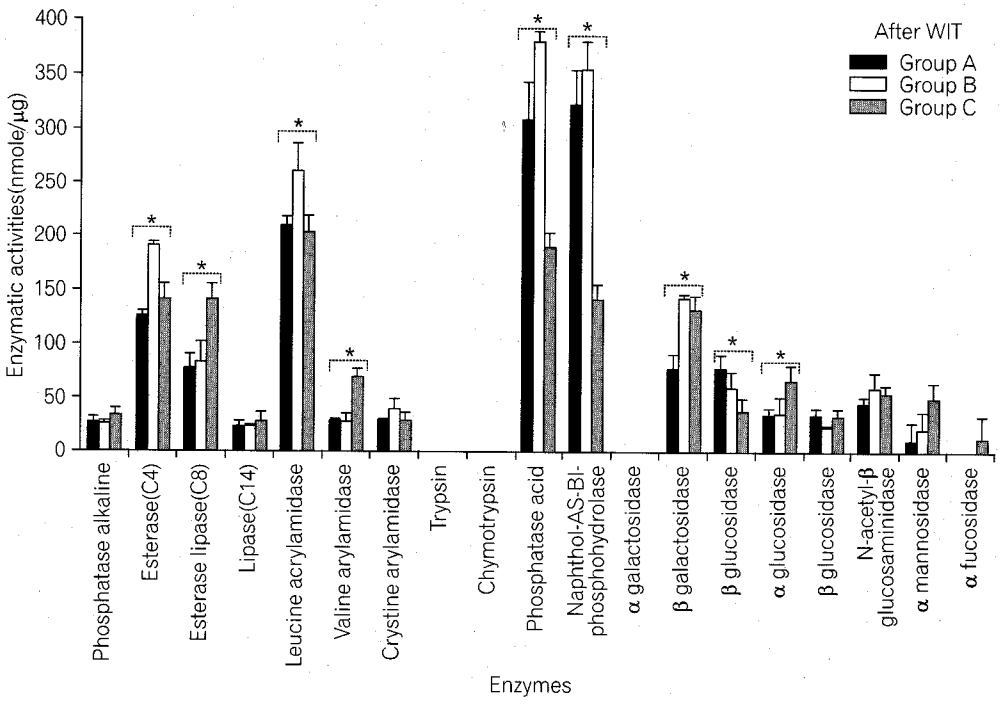

Fig. 2. The determination of enzymatic activities with various warm ischemic times: ($p<0.05$, significantly different between groups in each enzyme by one-way ANOVA).*

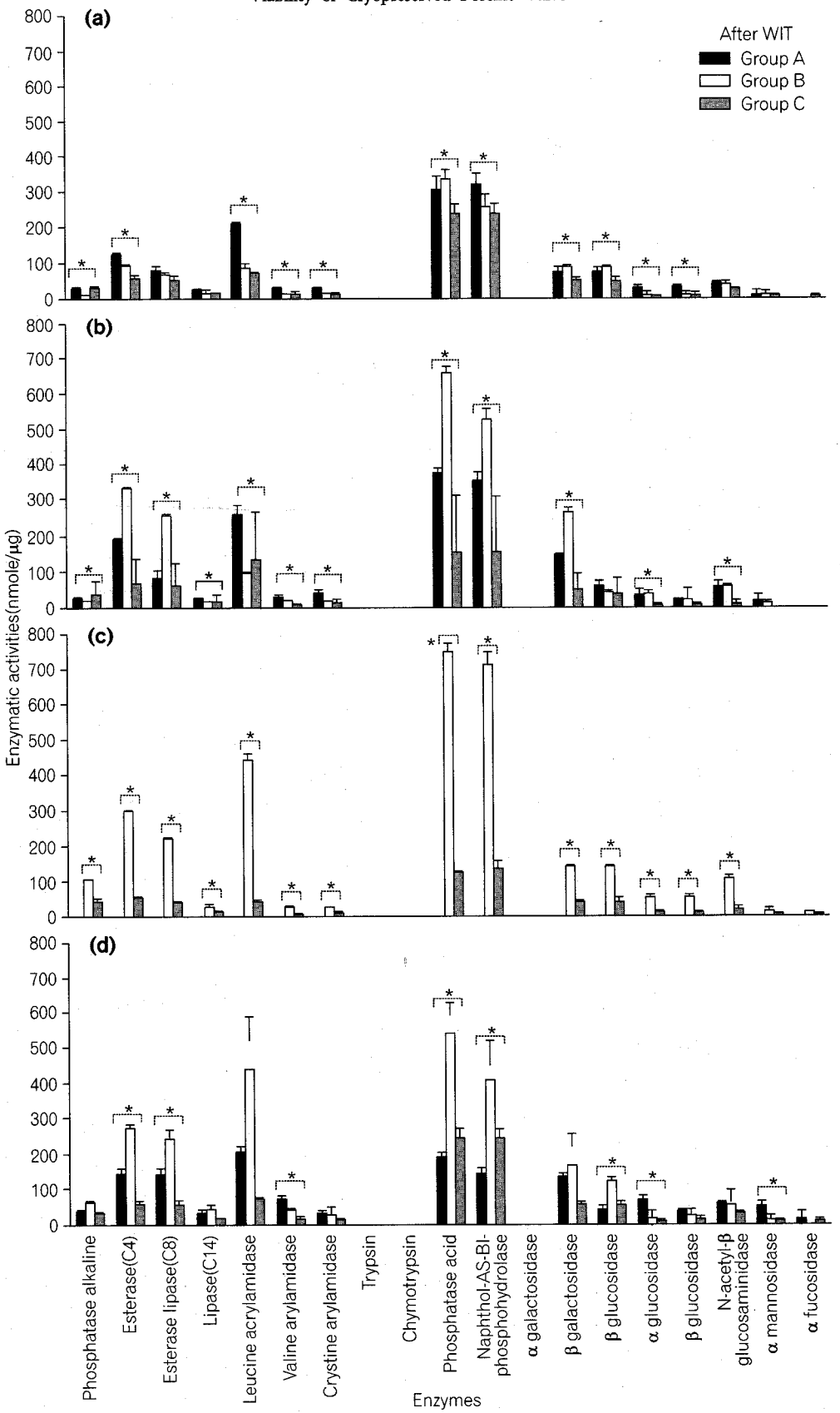

Fig. 3. The determination of enzymatic activities after cold ischemia, and cryopreservation. (a) group A, WIT=2, (b) group B, WIT=12, (c) group C, WIT=24 hours, and (d) group D, WIT=36. ($p<0.05$, significantly different between preservation processes in each enzyme by one-way ANOVA or by t-test).*

noted between the WIT and fibroblast viability ($r=-0.856$). Cold ischemia for 24 hours also had an influence on the viability significantly in each group ($p<0.05$). Viability was reduced remarkably after CIT, followed by 12 hours of WIT (group B), while the fibroblast viability in leaflets between after- CIT and after-cryopreservation revealed no statistically significant differences ($p=0.656$).

The activity changes of 19 enzymes after WIT, CIT and cryopreservation were observed. As WIT increased, esterase (C4), leucine arylamidase, phosphatase acid, phosphohydrolase and b-galactosidase significantly increased for up to 12 hours of WIT and were then reduced (Fig. 2). Fig. 3 shows that several enzymes were similarly activated or deactivated according to each preservation process in groups A-D. The activities of esterase (C4), esterase lipase (C8), phosphatase acid, and phosphohydrolase were significantly different in all groups. These enzymes were activated after CIT and then deactivated after cryopreservation in groups B, C, and D. In addition, enzymatic activities in group D were reduced significantly in comparison to group C after CIT (Fig. 3, c and d).

DISCUSSION

It has been suggested that the clinical durability of cryopreserved bioprosthetic tissue valves depends on the fibroblast viability of the valves after implantation.[6,10,13] WIT is thought to be an important determinant of fibroblast viability. This study observed the changes of fibroblast viability in heart valves in relation to WIT, cold ischemia and cryopreservation using trypan blue staining, which has traditionally been used to determine the viability of cells. Trypan blue dye is excluded by viable cells, but it is up-taken by nonviable cells due to the loss of cell membrane integrity.[23] In addition, the changes of enzymatic activities were also investigated to confirm the viability test results from the trypan blue dye exclusion. API ZYM kit was used to assess the activity of various enzymes related to the intracellular metabolism. These enzymes are chiefly lysosomal hydrolases.[24]

Fig. 1 shows a significantly negative correlation between WIT and cell viability, and demonstrates that extending WIT decreases the viability of fibroblasts. Cold ischemia for 24 hours did not produce a significant change of enzymatic activities after 2 hours of WIT. Although the cellular mechanism involved in cold ischemia is unclear, it has been reported that cold ischemia for 24 hours with less than 12 hours of WIT did not have a significant effect on the cellular metabolism.[18,25] However in this study, it was observed that cold ischemia influenced both the cellular viability and enzyme activity of leaflet tissue in cases of more than 12 hours of WIT. This result indicates that valves with less than 12 hours of WIT should be transported within 24 hours in cold storage condition.

The decreased fibroblast viability led us to assume that cold storage for 24 hours results in the loss of cell membrane integrity. This was consistent with the marked activation of enzymes related to lipid degradation, for these enzymes disrupt cell membrane (Fig. 3b). Particularly, phosphatase acid and phosphohydrolase were activated remarkably, and this would then be related to the degradation of nucleic acids and increased consumption of high energetic phosphates such as ATP etc. as a result of initial tissue damage. After ischemia, leaflet cellular metabolism shifts from aerobic to anaerobic, and thereby depletes part of leaflet cells' metabolic reserves, thus leading to a reduction in energy dependent cell functions such as proliferation, protein transport, and synthetic activity. As a result, the cells lose their membrane structure, start the degradation of nucleic acid, and lead to cell death.[4,19,26] However, it was further studied as to which enzyme was used as a sensitive marker to determine the damage of leaflet tissue following storage.

In observation of the viability of fibroblast after WIT, group C (WIT=24 hours) revealed a significant decrease, but group B (WIT=12 hours) showed a remarkable reduction after 24 hours of cold ischemia. This result suggests that the favorable warm ischemic tolerance time before implantation is up to 24 hours without considering cold storage, but it is less than 12 hours considering 24 hours of CIT. This was consistent with some reports that the use of valves harvested more than 24 hours after the donor's death may be unwise because the valve leaflet cells would be nonviable.[17,21] However, it is still uncertain how much fibroblast viability is required for successful implantation. When warm ischemic time is extended to 36 hours, the activity of enzymes was decreased more (Fig. 2 and 3, c and d), and this indicated that cells face irreversible and more severe damage than in WIT condition after 24 hours, and therefore die.[4] Though enzymatic activities were not performed on

group C after WIT, it could be predicted that the activities would be in correlation with the fibroblast viability. After cryopreservation, fibroblast viability was maintained as much as after CIT in trypan blue dye exclusion (Fig. 1). This result supports the brief that the use of liquid nitrogen for tissue storage is acceptable, and it is in agreement with the report that a heart valve leaflet stored at $-196^{\circ}C$ has been shown to be stable for at least 10 years.[8,15] However, enzymatic activity decreased after cryopreservation in all the groups, and it suggested that intracellular enzymes, though viability was maintained, were deactivated during cryopreservation (Fig. 3).

In conclusion, the shorter WIT was, the more effectively the viability of fibroblast could be mainteined. Cold ischemic condition for 24 hours provided changes in both fibroblast viability and enzymatic activity of leaflets on WIT less than 12 hours, but remarkably influenced them on WIT of more than 12 hours. Cryopreservation did not significantly affect the viability of fibroblasts. Therefore, it is suggested that a procured valve has to be transported and cryopreserved within 12 hours of WIT in consideration of 24 hours of cold ischemia.

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