# Flow Cytometric Analysis of Endothelial Cell Viability in Arterial Allograft

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=Abstract=

## 동종동맥판 혈관내피세포의 생육성 평가에 관한 연구

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Arterial allografts have known advantages over prosthetic vascular conduit for treatment of heart valvular disease, congenital heart disease and aortic disease. Cell viability may play a role in determining the longterm outcome of allografts. Endothelial cell is one important part in determining the allograft viability.

To evaluate the viability of endothelial cells using current allograft preservation technique, porcine heart valve leaflets and arterial wall were subjected to collagenase digestion. Single endothelial cell suspension was labeled with GSA-FITC(Griffonia simplicifolia agglutinin-fluorescein isothiocyanate), a vascular endothelial cell specific marker. The cell suspension was washed and incubated with PI(Propidium Iodide), which does not bind with viable cells. Endothelial cell viability was evaluated by calculating the percentage of GSA-FITC(+) and PI(-) group using flowcytometric analysis. Allografts were treated with 4℃ antibiotic solution for 24 hours for sterilization. After this, half of allografts were stored in 4℃ RPMI 1640 with HEPES buffer culture medium with 10% fetal bovine serum for 1 to 14 days(Group I). Another half of allografts were cryopreserved with a currently used technique (Group II). During the procurement and sterilization of arterial allografts, 22.8% and 24.4% of endothelial cell viability declined, respectively. In Group I, 11.9% of endothelial cell viability declined further steadily during 14 days of storage. In Group II, 13.7% of endothelial cell viability declined. These results show that largest loss of endothelial cell viability occurs during the initial process. After 14 days of arterial allograft storage under 4℃ nutrient medium or cryopreservation, about 40% of endothelial cell viability is maintained. There were no differences between the endothelial cell viability from aortic valve leaflet, pulmonic valve leaflets, aortic wall and pulmonic wall.

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**Key words** : 1. Allograft
2. Endothelial cell, vascular
3. Viability

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## INTRODUCTION

Arterial allograft has been widely used for heart valve replacement, conduit replacement of aortic root, right ventricular outflow tract reconstruction, and great arterial replacement.

The long term survival of allograft appears to be limited and cellular viability seems to be a factor in determining its longevity. The determination of cellular viability after hypothermic storage at 4℃ and cryopreservation is important to improve preservation technique and its clinical results. Although there may be many factors in determining the cellular viability, the endothelial cell may be an important part in determining the viability of allograft.

This study was performed to quantify the viability of endothelial cells of arterial allograft after storage with currently clinically using technique, 4℃ hypothermic storage and cryopreservation, using flow cytometric technique in the porcine model.

## MATERIAL AND METHODS

Porcine hearts with ascending aorta and pulmonary artery were obtained from the slaughterhouse immediately after the animals were killed and delivered to the laboratory in 4℃ cold saline. Ascending aorta and pulmonary artery trunk with an intact valve, myocardial cuff and anterior mitral leaflet were excised. Portions of the ascending aorta, aortic valve, pulmonary artery and pulmonic valve were excised and sent for the flow cytometric analysis of viable endothelial cells(D0). Grafts were then placed in 4℃ RPMI 1640 with HEPES buffer culture medium with 10% fetal bovine serum and the following antibiotics: Cefoxitine 240mg/l, Lincomycin 120mg/l, Polymixin B 100mg/l, Vancomycin 50mg/l. After 24 hours, grafts were rinsed with fresh medium without antibiotics and divided into two groups. Group 1: After excision of some tissue from arterial wall and valve leaflet of aorta and pulmonary artery, grafts were transfered to 4℃ RPMI 1640 with HEPES buffer culture medium with 10% fetal bovine serum. Excised tissues were sent for the viability assay(D1). The grafts were maintained in this solution at 4℃ for 1, 2, 7, 14 days and the tissues from same part of the grafts were sent for the viability assay at the designated time(D2, D3, D7, D14), respectly. Group 2: After excision of some tissue for viability assay(D1), the grafts were placed in 4℃ RPMI 1640 with HEPES buffer culture medium, 10% fetal bovine serum and 10% DMSO solution (freezing medium). The grafts were frozen in a freezing chamber (CryoMed 1010, Mount Clemens, Michigan, USA) at the controlled freezing rate of −1℃ per minute utilizing a programmable microcomputer controller. As the graft temperature reached −40℃, grafts were transferred to the liquid nitrogen vaporphase temperature storage unit(−196℃) and stored there for 14 days. After 14 days of storage, grafts were placed in 40℃ water bath for about 11 min for rapid and complete thawing and then stepwise dilution of DMSO and reequlibriation of allograft was followed for 5min. After this, same portion of graft were excised from arterial wall and valves of graft and sent for viability assessment(D14cryo).

We performed flow cytometric analysis of endothelial cells to assess the cellular viability of graft. A piece of allograft wall or valve leaflet was placed in 0.1% collagenase type 1A(Sigma Chemical Co, St. Louis, MO, USA) and incubated at 38℃ for 10 minutes. The luminal surface of the graft was scrubbed gently with a glass rod to separate the endothelial cell layer. The collagenase solution containing the endothelial cells was drained and centrifuged at 1,500 rpm for 5min. The supernatant was discarded and the pellet of endothelial cell was suspended in iced GSA-FITC(Griffonia simplicifolia agglutininfluoresein isothiocyanate) solution (2g/ml) for 30 minutes. Cells were washed in phosphate buffered saline and centrifuged three times to washout the unattached GSA-FITC. Cells were then suspended in PI(Propidium Iodide) solution(50g/ml) for 3 minutes. Just after the 3 minutes of treatment in PI solution, cells were placed in a pressurized cell suspension chamber and passed through a 67 m Millipore filter into the flow chamber. The cells were passed through argon ion laser. The emitted light passed through a 525-nm bandpass filter and 635-nm bandpass filter for GSA-FITC and PI analysis. The data was then sorted and intregrated by a fluorescence-activated cell sorter (FAC Star Plus, Becton & Dickinson, USA). At least 10,000 cells were sorted for each specimen. GSA has specific affinity to the $\alpha$-D-galactopyranosyl residue of vascular endothelial cell and GSA-FITC(+) group consists of pure endothelial cells. PI has specific affinity to the double stranded nucleic acid and can not attach to the living cells with intact cell membrane because PI can not penetrate through the intact cell membrane. PI(−) group consisted of living cells. Cells that were GSA-FITC(+) and PI(−) were considered viable endothelial cells and GSA-FITC(+) and PI(+) were nonviable endothelial cells(Fig. 1). The proportion of GSA-FITC(+) and PI(−) cells from all GSA-FITC

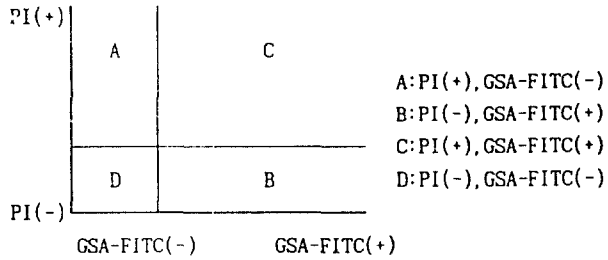


**Fig. 1.** Flow cytometric analysis of endothelial cell viability of aortic valve leaflet at procurement. Percent gated cell of none B,C is 70.81% abd 14.09%, respectively. Percent of viability=B / B+C=83.4% Zone A: non-viable, non-endothelial cell group, Zone B: viable endothelial cell group, Zone C: non-viable endothelial cell group, Zone D: non-significant zone

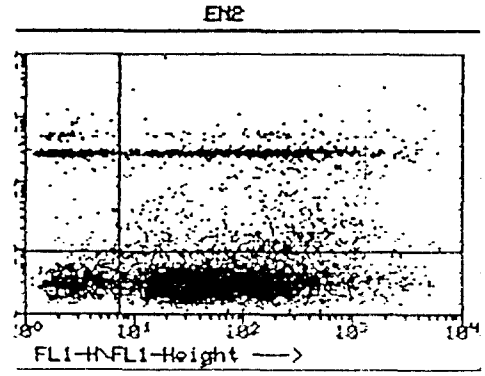


**Fig. 2** Endothelial cell viability of aortic wall(#1) , aortic valve leaflet(#2), pulmonary artery wall(#3), pulmonary valve leaflet(#4) during the storage in 4 C nutrient medium.
D0: at procurement, D1: after sterilization, D2: after 1 day storage, D3: after 2 days storage, D7: after 7 days storage, D14: after 14 days storage

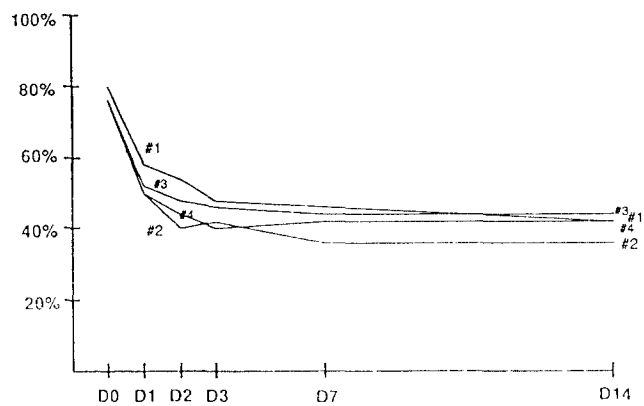


**Fig. 3** Endothelail cell viability of aortic wall( #1), aortic valve leaflet( #2), pulmonary artery wall( #3), pulmonary valve leaflet(#4) after the cryopreservation.
D0: at procurement, D1: after sterilization, D14: after 14 days storage

(+) cells were recorded as viability of vascular endothelial cells. The mean and standard error was recorded for each specimens.

For statistical analysis of endothelial cell viability results, comparision were performed using one way ANOVA test. When significant differences were found, multiple t-test were performed. A value $<0.05$ was considered statistically significant.

## RESULT

Results are reported as mean percentage of viable endothelial cells standard error. The endothelial cell viability of aortic wall(AW), aortic valve(AV), pulmonic wall(PW) and pulmonic valve(PV) from control group(at harvest: D0) was 80.0±15.9%, 76.3±10.7%, 76.3±8.2%, 76.1±4.4%, respectively. The viability of endothelial cell of AW, AV, PW, PV after treatment in antibiotic solution for 24 hours(D1) was 58.0±10.7%, 50.4±1.9%, 52.2±6.5%, 50.4±10.5%, respectively. After storage of 24 hours(D2) in Group 1, the viability of endothelial cell of AW, AV, PW, PV was 54.0±9.3%, 41.3±6.6%, 49.0±7.3%, 44.0±2.1%, respectively. After storage of 48 hours (D3) in Group 1, the viability of endothelial cell of AW, AV, PW, PV was 47.5±4.6%, 42.3±7.6%, 45.2±5.4%, 40.3±6.9%, respectively. After storage of 7days(D7) in Group 1, the viability of endothelial cell of AW, AV, PW, PV was 45.8±1.2%, 36.9±3.3%, 43.7±1.5%, 41.9±3.1%, respectively. After storage of 14days(D14) in Group 1, the viability of endothelial cell of AW, AV, PW, PV was 41.4±4.8%, 36.5±12.4%, 43.5±2.7%, 41.6±4.5%, respectively(Fig. 2). After cryopreservation for 14 days (Dcryo) in Group 2, the viability of endothelial cell of AW, AV, PW, PV was 37.7±4.9%, 39.9±3.4%, 34.9±2.4%, 43.7±2.5%, respectively(Fig. 3). There was significant negative correlation between duration of storage and endothelial cell viability in Group 1 and 2($p < 0.05$; Fig. 4).

## COMMENT

The allograft has been used widely in treatment of heart valvular disease and great arterial disease, recently. Cellular viability of allografts has been generally considered an important factor in determination of longterm survival of graft[1,2]. Endothelial cells and

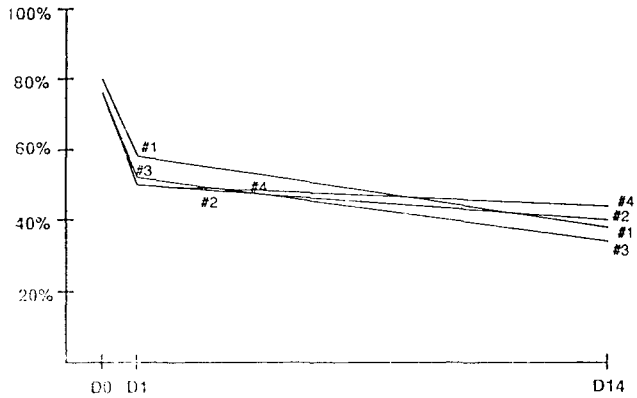


**Fig. 4.** Change of endothelial cell viability after the cryo- preservation (#1) and the storage in 4℃ nutrient medium(#2).
D0: at procurement, D1: after sterilization, D2: after 1 day storage, D3: after 2 days storage, D7: after 7 days storage, D14: after 14 days storage

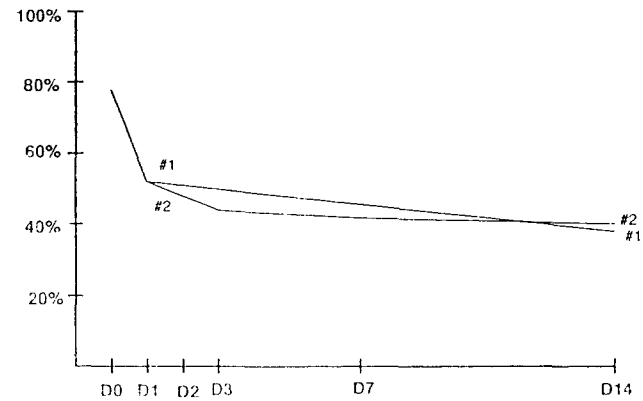


**Fig. 5.** Change of endothelial cell viability after the cryo-preservation(#1) and the storage in 4℃ nutrient medium(#2),
DO : Atprocurement. D1 : after sterilization.
D2 : afte 1 day storage. D3 : after 2 days storage.
D7 : after 7 days storage. D14 : after 14 days storage

fibroblasts are the major cellular components of allograft. The endothelial cell lines vascular tree and is the interface between graft and host tissue. Functions of endothelial cell include resistance to thrombosis, maintenance of hemostasis, and modulation of vascular smooth muscle activity. Viable endothelial cellsmay be important in long term survival of graft by providing nutrition to the fibroblast, prevention of thrombosis, inhibition of calcification[3,4].

Viability of allograft endothelial cells may be affected by ischemic time before preservation(warm ischemic time)[5], preservation methods including sterilization and storage[5~7]. Mochtar and associates found that fibroblast viability declined markedly after 2 weeks of storage under 4℃[8] and fibroblast has been considered as the most important factor in determining allograft viability. Christy and associates found endothelial cell viability declined progressively during 2 weeks of storage in 4℃ nutrient medium[9]. Although the interrelation of these two cells are not clearly defined, endothelial cell viability may play an important role in determining the allograft survival in respect to cellular viability and graft antigenicity. Yankah and associates demonstrated a progressive decline of endothelial cell viability during storage in 4℃ nutrient medium using Alcian blue dye exclusion technique in rat5 and human model[7].

Flow cytometry is useful in the determination of endothelial cell viability compared to other techniques like dye exclusion technique or metabolism assay because it provides precise quantative analysis through identification of viable cell among single cell suspensions. Griffonia simplicifolia is a kind of lectin and has a high affinity for the $\alpha$-D-galactopyranosyl residues of vascular endothelial cells. Its conjugation to fluorescein permits rapid evaluation of large number of cells using automated method[10]. Propidium iodide is a dye with high affinity for double stranded nucleic acids and excluded by viable cells with intact cell emebrane function[11]. Using these two differentstains, one staining only endothelial cells and one staining only dead cells, flow cytometer counts endothelian cells and dead cells independently.

In this study, endothelial cell viability declined 22.8%(20.0%~23.9%) during the harvesting of allograft and there was no statistical difference of viability between the endothelial cells from aortic wall, aortic valve, pulmonic wall and pulmonic valve. After the treatment of allograft in 4℃ antibiotic solution for 24 hours, endothelial cell viability declined 24.4%(22.0%~25.9%). During the allograft preservation in 4℃ nutrient medium for 14 days, endothelial cell viability was reduced 5.7%(3.2%~9.1%), 8.9%(7.0%~10.5%), 10.6%(8.5%~13.5%), 11.9%(8.4%~16.6%) after 1, 2, 7, 14 days of storage, respectively. After cryopreser- vation of allograft for 14 days, endothelial cell viability declined 13.7%(6.7%~20.3%) and there was no statistical differences between the tissues at each designated time.

These results show that the endothelial cell viability declines abruptly(47.2%) during the initial harvesting and sterilization process and steadily thereafter during the later periods of preservation in 4℃ nutrient medium. These findings are comparable to the results of

Christy and associates[9], Lupinetti and associates[12] showed steady decline of endothelial cell viability over the storage period. 40.9% of viable endothelial cell was preserved after 14days of 4℃ cold storage of allograft, and similar decline in endothelial cell viability after cryopreservation was found. Only 13.7% of endothelial cell viability declined during the cryopreservation period. Yankah and associates were the first to examine the presence and viability of allograft valve endothelium in a rat mode[15]. They discovered cryopreserved valved contained 8~20% less viable endothelial cells than those of untreated valve grafts[13]. Lupinetti and associates discovered that endothelial cell was observed on 16% of cryopreserved allograft[14].

To preserve better endothelial cell viability, short and strict harvesting process to reduce the warm ischemic time and optimal sterilization protocol need to be defined. Viable endothelial cell may, however, contribute to increased antigenicity of allografts because the endothelium is an important site of antigen expression[15]. Although many factors are concerns in terms of allograft longevity, the role of viability of endothelial cells together with that of fibroblast must be defined first. The present study may be used to investigate the relationship between endothelial cell viability and allograft immunogenicity.

There were no differences of viability between the endothelial cells from arterial wall and valve,or aorta and pulmonary artery, suggesting that endothelial cells either from arterial wall and valve leaflet were damaged at a similar rate during the storage.

Flow cytometry is useful in the evaluation of endothelial cell viability because it provides precise and quick quantitative analysis compared to other methods. It is believed that this technique can be used in further similar investigation of arterial allograft involving animal and human models.

## References

1. OBrien MF, Johnston N, Stafford G. *A study of the cells in the explanted viable cryopreserved allograft valve.* J Cardiac Surg 1988; 3(Suppl): S279-87.
2. Ross D. *The versatile homograft and autograft valve.* Ann Thorac Surg 1989;48:S69-70.
3. Jaffe EA. *Cell biology of endothelial cells.* Hum Pathol 1984;18:234-9.
4. Wolfinbarger L Jr, Hopkins RA. *Biology of heart valve preservation.* In: *Cardiac reconstructions with allograft valves,* Hopkina RA(eds). Springer-Verlag: New York, pp 31, 1989.
5. Yankah AC, Randzio G, Wottge HU, Bernhard A. *Factors influencing endothelial-cell viability during procurement and preservation of valve allografts. In: Microsurgical models in rats for transplantation research,* Thiede A, Deltz E, Engemann R, Hamelmann H(eds). Springer-Verlag: Berlin, pp 109, 1985.
6. Innes BJ, Thomson NB Jr, Ayers W. *Postmortem changes in endothelial cells of aortic valve homograft.* J Thorac Cardiovasc Surg 1969;58:416-23.
7. Yankah AC, Wottge H-U, Muller-Hermelink HK. *Transplantation of aortic and pulmonary allograft, enhanced viability of endothelial cells by cryop reservation, importance of histocompatibility.* J Cardiac Surg 1987;1(Suppl):209-20
8. Mochtar B, van der Kamp AWM, Roza-De Jongh EJM, Nauta J. *Cell survival in canine aortic heart valves stored in nutrient medium.* Cardiovasv Res 1984;18: 497-501
9. Christy JP, Lupinetti FM, Mardan AH, Thompson SA. *Endothelial cell viability in the rat aortic wall.* Ann Thorac Surg 1991;51: 204-7.
10. Peters BP, Goldstein AJ. *The use of fluorescein-conjugated Bandeiraea simplicifolia B4-isolectin as a histochemical reagent for the detection of α-D-galactopyranosyl groups.* Exp Cell Res 1979;120: 321-34.
11. Krishan A. *Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining.* J Cell Biol 1975;66:188-93.
12. Lupinetti FM, Christy JP, King DM, Khatib HE, Thompson SA. *Immunogenicity, antigenicity, and endothelial viability of aortic valves preserved at 4℃ in a nutrient medium.* J Cardiac Surg 1991;6:454-61.
13. Yankah AC, Dreyer W, Wottge H-U, Muller-Rucholtz, Bernhard A *Kinetics of endothelial cells of preserved aortic valve allografts used for heterotopic transplantation in inbred rat strains.* In: *Biologic and bioprosthetic valves,* Bodnar E and Yacoub MH(eds). Yorke Medical Books: New York, pp 73-84, 1986.
14. Lupinetti FM, Tsai TT, Kneebone JM, Bove EL. *Effect of cryopreservation on the presence of endothelial cells on human valve allografts.* J Thorac Cardiovasc Surg 1993;106:912-7.
15. Pobers JS, Collins T, Gimbrone MA Jr, Libby P, Reiss CS. *Inducible expression of class II major histocompatibility complex antigens and immunogenicity of vascular endothelium.* Transplantation 1986; 41:141-6.

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**=국문초록=**

동종동맥판은 심장판막질환, 선천성 심기형 및 대동맥 질환의 치료에 있어서 우수한 판막도관으로 사용되고 있다. 이 때 동종동맥판의 장기성적을 좌우하는데 있어서 혈관내피세포의 생육성이 중요한 역활을 할 것이다.

혈관내피세포의 생육성을 평가하기 위하여 현재 임상에서 사용되는 보존방법으로 보존처리된 성돈의 대동맥판 및 대동맥벽을 collagenase로 분해시켜서 순수한 내피세포군을 획득한 뒤, 혈관내피세포에 특이한 친화성을 갖는 GSA-FITC(Griffonia simplicifolia agglutinin- fluorescein isothiocyanate)와 반응시켰다. 이 내피세포군을 세척한 다음, 살아있는 세포에는 침착되지 않는 PI(Propidium Iodide)와 반응시켰다. 이렇게 처리된 내피세포군을 Flow Cytometry 로 분석하여 GSA-FITC(+), PI(−) 인 세포를 생육성을 유지한 것으로 평가하였다.

동종동맥판은 4℃의 멸균용액에 24시간 담궈 멸균처리를 한 후, 2개군으로 나누어(1군)은 4℃ RPMI 1640 with HEPES buffer culture medium with 10% fetal bovine serum 용액에 1~14일간 보존하였고 (2군)은 냉동보존을 하였다. 조직의 획득과정과 멸균과정에서 각각 22.8%와 24.4%의 생육성이 소실되었다. (1군)에서는 14일의 보존기간 동안 11.9%의 생육성감소가 일어났고(2군) 에서는 13.7%의 생육성감소가 일어났다.

이 실험의 결과로 동종동맥판의 보존처리과정 초기에 대부분의 생육성소실이 일어나며, 14일간의 냉장보존이나 냉동보존 후에도 약 40%의 생육성이 보존됨을 알 수 있었다. 또한 혈관내피세포가 판막에서 얻어진 경우나 동맥벽에서 얻어진 경우에서 생육성의 차이는 없었다.

**중심단어 :** 1. 동종조직
2. 혈관내피세포
3. 생육성