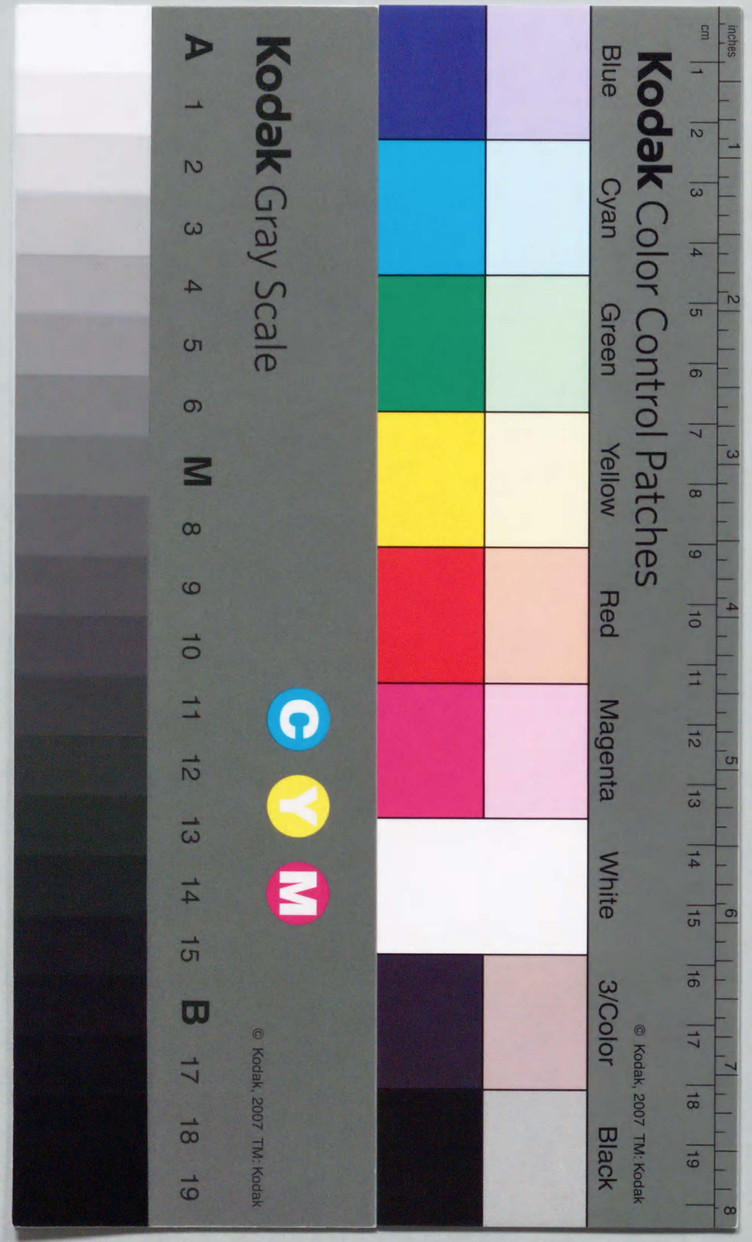

主論文

A Novel Strategy of Decoy Transfection Against Nuclear Factor-kB in Myocardial Preservation

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Background. Nuclear factor-kB (NFkB) is critical for the transcription of multiple genes involved in myocardial ischemia-reperfusion injury. Therefore, we hypothesized that blocking NFkB would attenuate ischemia-reperfusion injury after prolonged myocardial preservation, resulting in an improvement of cardiac function.

Methods. Double-stranded oligonucleotides (ODNs) with a specific affinity for NFkB (NFkB decoy; NF group) or a scrambled decoy (SD group) were transfected into rat hearts using a hemagglutinating virus of Japan (HVJ)-liposome method. After a 16-hour 4 °C preservation in Euro-Collins solution, the cardiac grafts were heterotopically transplanted into the recipient rat of same strain.

Results. FITC staining showed introduction of ODNs into the nuclei of endothelial cells and cardiomyocytes. After 1 hour of reperfusion, the NF group showed significantly higher recoveries of left ventricular function as well as significantly lower levels of serum creatine phosphokinase, myocardial water content, tissue IL-8, and neutrophil infiltration than the SD group ($p < 0.05$).

Conclusions. Gene transfection of the NFkB decoy attenuates ischemia-reperfusion injury after prolonged heart preservation. As a result, this method appears to be a novel strategy for enhanced myocardial preservation.

(Ann Thorac Surg 2000, in press)

Cardiac transplantation is now recognized as an established therapy for irreversible and final stage heart failure. However, preservation of hearts is a major limitation in clinical heart transplantation. The main cause of death early after transplantation seems to be ventricular dysfunction, rather than rejection or infection, resulting from inadequate preservation [1]. Several strategies have been developed to improve organ preservation and attenuate reperfusion injury to the graft [2-6]. However, they remain still inadequate to attenuate contractile and coronary dysfunction

In the process of ischemia-reperfusion injury in the myocardium, endothelial cell activation is an initial step and plays a key role in the induction of adhesion molecules and cytokines [6-8]. In endothelial cell activation, nuclear factor kappa-B (NFkB) is the main transcription factor involved in up-regulation of pro-inflammatory gene products [9,10]. The expression of these genes plays an important role in the initiation of inflammatory response by targeting circulating leukocytes at the site of inflammation. Therefore, blocking NFkB in the endothelial cells seems to be a useful strategy for attenuating ischemia-reperfusion injury in myocardium

Synthetic double-stranded oligodeoxynucleotides (ODNs), as "decoy" *cis* elements, block the binding of nuclear factors to the promoter regions of targeted genes, resulting in the suppression of gene transactivation in *in vitro* and *in vivo* models [9,10]. We have previously demonstrated that an *in vivo* transfection of an NFkB decoy ODN using hemagglutinating virus of Japan (HVJ)-liposome as a vector attenuates ischemia-reperfusion injury in rat hearts [9,10].

In this study, we hypothesized that transfection of NFkB decoy into endothelial cells and myocytes during hypothermic storage would enhance myocardial preservation by attenuating leukocyte-mediated reperfusion injury. The aim of this study is to examine the efficacy of decoy transfection for enhanced myocardial preservation.

Methods

Animals.

Adult male Sprague-Dawley rats were used in this study. All animals received humane care in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No.85-23, revised 1985).

Synthesis of ODN.

The sequence of the phosphorothioate ODNs utilized (NFkB decoy ODN and Scrambled decoy ODN) were previously described [9,10]. NFkB decoy ODN has been shown to bind the NFkB transcriptional factor [11]. For histological assessment of the efficiency of the ODN delivery, NFkB decoy ODNs were labeled with FITC on the 3′ end using fluorescein-ODN phosphoramidite. These ODNs were provided by Greiner Japan Co. (Tokyo, Japan).

Ex vivo transfer of ODN using the HVJ-liposome method.
HVJ-liposome was prepared as previously described [12,13]. Sprague-Dawley rats (250 g) were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg). After anticoagulation with heparin (200 USP units, intravenously), the hearts were arrested with cold crystalloid cardioplegic solution infused via the aorta. The hearts were excised and infused with 0.7 ml of HVJ-liposome–ODN complex via the ascending aorta, with the vena cavae, pulmonary artery, and veins ligated. After incubation on ice for 10 min, the hearts were stored in 4 °C Euro-Collins solution for 16 hours with the pulmonary artery open [10,14].

Heterotopic cardiac transplantation
Recipient rats (350g) of the same strain were anesthetized with the same way as the donor rats. For the procedure, each rat was placed on a warm water mat to maintain the rectal temperature at approximately 37 °C, which was continuously monitored, and continuously provided with 0.5 L/min of 100% O_2. The left femoral artery and vein were cannulated for simultaneous monitoring of blood pressure and heart rate, as well as volume substitution. The abdomen was opened with a midline incision and the donor heart was transplanted after hypothermic storage by means of a modification of the Ono and Lindsey technique [15]. After completion of the anastomoses, the heart was then reperfused with blood in situ for 1 hour [6,16].

Histological assessment
A histological assessment of the efficiency of the FITC-labeled ODN delivery by HVJ-liposome method in comparison with a direct transfer was performed. Rat hearts were transfected with FITC-labeled ODN with HVJ-liposome (10 μM, n=6) or without HVJ-liposome (10 μM, n=6). The hearts in each group were taken after 16 hours of preservation (n=3 in each group) or after preservation followed by 1 hour of reperfusion (n=3 in each group). They were frozen at –80 °C, cut into thin sections (5 μm), and then examined by fluorescence microscopy.

Cardiac graft function and serum creatine phosphokinase
The performance of the transplanted heart was measured in situ after 1 hour of reperfusion. A thin-walled latex balloon tipped catheter was introduced into the left ventricle via the left atrial appendage to monitor left ventricular pressure. After 1 hour of reperfusion, heart rate (HR), left ventricular developed pressure (LVDP), and the maximal derivatives of left ventricular pressure (max dP/dt) were measured with the left ventricular end-diastolic pressure stabilized at 10 mmHg. Crystalloid volume substitution (Ringer's solution) was adjusted via the left femoral vein to maintain the mean arterial pressure of the recipient rat between 65-80 mmHg for the reperfusion period. After completion of reperfusion, the hearts was excised and immediately frozen at –80 °C for histological assessment. Aortic blood samples were collected for the assay of serum creatine phosphokinase (CPK).

Myocardial water content.
To evaluate myocardial water content after the preservation followed by reperfusion, the basal region of the heart was taken and weighed. Next, it was desiccated at 96 °C for 24 hours and then reweighed. The myocardial water content was calculated by the following formula:

Myocardial water content =
(1 – dry weight / wet weight) X 100 (%)

Neutrophil adherence and tissue IL-8
Neutrophils in the myocardium were selectively stained using a naphtol AS-D chloroacetate esterase kit (Sigma, St.Louis, MO, USA). The number of neutrophils was counted under light microscopy (magnification, X 100) in a blind manner. The total number of neutrophils in each section was summed and expressed as cell number per section. At least ten individual sections were evaluated per heart.
For measurement of tissue IL-8 production, the transfected hearts were excised after preservation followed by reperfusion and immediately frozen in liquid nitrogen. The samples were homogenized with a Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY) and centrifuged. The concentration of tissue IL-8 was measured using ELISA kits (Immuno-Biological Laboratories Co. Gumma, Japan) according to the manufacturer's recommendations.

Study Group
Hearts were divided in two groups (n = 6 in each group): the NF group, transfected with the *cis* element "decoy" against the NFkB binding site, and the SD group, transfected with the scrambled decoy ODN.

Statistical analysis
All data are expressed as mean ± standard error of the mean (SEM). Scores were compared using an unpaired Student's *t* test. P values of less than 0.05 were considered statistically significant.

Results

Ex vivo transfection of NFkB decoy ODN
Fluorescence was detected in microvascular endothelial cells of hearts transfected with FITC-labeled ODN by the HVJ-liposome method after 16-hour of hypothermic preservation. Furthermore, after 1 hour of reperfusion, fluorescence was detected not only in vessels, but also in myocytes. The fluorescence was localized mainly in the nuclei of cardiac myocytes and endothelial cells. On the other hand, there was very little fluorescence detected in those hearts transfected with FITC-labeled ODN by direct transfer after both preservation and reperfusion (Figure 1). These results are consistent with those of our previous study [9,10].

Posttransplantation cardiac function and serum CPK
There were no significant differences in the recipient hemodynamic parameters between the two groups (Table 1). Systolic functional recoveries after 1 hour of reperfusion in terms of LVDP (NF vs. SD, 57 ± 5 vs. 21 ± 9 mmHg; P < 0.05) and max dP/dt (1225 ± 154 vs. 235 ± 76 mmHg/sec; P < 0.01) were significantly better in the NF group than in the SD group, whereas there was no significant difference in HR between both groups (Figure 2).
Serum CPK levels were significantly lower in the NF group than in the SD group (3943 ± 834 vs. 7170 ± 367 IU/L; P < 0.05) (Figure 3).

Myocardial water content
The myocardial water content in the NF group was significantly lower than in the SD group (78.5 ± 0.6 vs. 80.5 ± 0.4 %; P < 0.05).

Neutrophil adherence and tissue IL-8
Cytohistochemical staining for neutrophils showed significant blockage of migration and/or accumulation of neutrophils into the myocardium in the NF group, compared to the SD group (9.7 ± 0.6 vs. 14.9 ± 0.9 counts/field with magnification X100; P < 0.01)(Figure 4).
The tissue level of IL-8 was significantly lower in the NF group than in the SD group (1850 ± 161 vs. 2614 ± 18 ng/mg tissue; P < 0.01)(Figure 5).

Comment

We herein report a novel therapeutic strategy to enhance myocardial preservation by attenuating ischemia-reperfusion injury, utilizing an *ex vivo* transfer of a *cis* element "decoy" ODN against the NFkB binding site. The mechanical function of hearts transplanted after 16 hours of hypothermic storage was significantly better preserved in hearts transfected with the NFkB decoy. In addition, those hearts transfected with the NFkB decoy showed significantly lower levels of serum CPK, myocardial water content, and neutrophils adherence accompanied by reduced tissue IL-8 production. These results suggest that NFkB plays a critical role in myocardial ischemia-reperfusion injury after prolonged preservation, and that blocking NFkB by gene transfection with a NFkB decoy is a novel therapy to enhance cardiac preservation by attenuating neutrophil-mediated reperfusion injury.
There have been several reports of *ex vivo* gene transfection into donor hearts during preservation by perfusion containing an adenovirus [17] or incubation with positive pressure [18]. Using a heterotopic rat heart transplantation model, we have previously reported the efficient gene transfer into cardiomyocytes of donor hearts with the HVJ-liposome method [14]. In the present study, it is noteworthy that delivery of FITC-labeled ODN into endothelial cells was demonstrated during the 16 hours hypothermic preservation without a following perfusion. This phenomenon may be attributable to HVJ adhering and fusing to endothelial cells under hypothermic condition. This seems to be important when considering the clinical settings of cardiac transplantation where access to the donor heart is allowed only at the time of harvest.

In myocardial injury following ischemia and reperfusion, endothelial cells have been shown to play an important role because they are anatomically located at the interface of blood and tissue exchange, and thus, are directly influenced by circulating leukocytes [6-8]. Under hypoxic circumstances, endothelial cells appear to be activated to express proinflammatory properties that include the induction of leukocyte-adhesion molecules and cytokines. This hypoxic endothelial cell activation leads to an accumulation of neutrophils in the endothelium and, as a consequence, amplifies reperfusion injury. IL-8 and ICAM-1 are regulated by the transcription factor NFkB, and both play especially important roles in neutrophil-mediated reperfusion injury. Several studies have shown that the inhibition of NFkB results in reduction of the enhancement of IL-8 and ICAM-1 expression [9,10]. In the present study, as well, the blocking of NFkB resulted in an inhibition of neutrophil adherence accompanied by the reduction of production of IL-8. In addition, our preliminary data showed a tendency of ICAM-1 expression in endothelial cells to be reduced not only after reperfusion, but also during the 16-hour hypothermic preservation in the hearts transfected with the NFkB decoy (data not shown). Although it is still controversial whether the expression of pro-inflammatory molecules could be up-regulated in hypothermic circumstances [19,20], NFkB was shown to be translocated to the nucleus at temperatures as low as 17 °C, even though active transcription did not take place [20]. Therefore, the transfected NFkB decoy ODNs might stabilize the phenotype of the microvessel cells so that the expression of pro-inflammatory molecules is suppressed during hypothermic storage, as well as during reperfusion. Further study is still required to clarify the mechanism and effects of the NFkB decoy on endothelial cell activation in hypothermic circumstances.
There have been several strategies to enhance cardiac preservation, including improvements in preservation solution [2,16], hypoxic preconditioning [3], and modification of reperfusion [4,5]. We have previously reported the efficacy of cardioplegia with leukocyte-depleted blood on dog heart grafts preserved for 24 hours, in which we focused on the importance of attenuation of neutrophil-mediated reperfusion injury [4]. An *ex-vivo* gene transfection of the NFkB decoy supports for this concept and seems to be potentially useful because it is technically simple and may act on endothelial activation during hypoxic preservation and following reperfusion.
In conclusion, an *ex vivo* gene transfection of the *cis* element "decoy" ODN against NFkB binding sites attenuated ischemia-reperfusion injury after prolonged heart preservation. As a result, this method appears to be a novel strategy for enhanced myocardial preservation, provided that vectors for gene transfection are proven safe for human.

Acknowledgements
We would like to thank Akiko Nishimura for excellent technical assistance in preparing HVJ-liposome.

References

1. Kaye MP. The registry of the International Society for Heart and Lung Transplantation: official report 1993. J Heart Lung Transplant 1993;12:541.
2. Gu K, Kin S, Saitoh Y, et al. Cardioprotective effect of nicorandil in histidine-tryptophan-ketoglurate solution during the cold storage of isolated hearts. Transplantation 1996;11:1572-5.
3. Engelman DT, Chen CZ, Watanabe M, et al. Improv0ed 4- and 6-hour myocardial preservation by hypoxic preconditioning. Circulation 1995;92(suppl):II417-22.
4. Fukushima N, Shirakura R, Nakata S, et al. Study of efficacies of leukocyte-depleted terminal blood cardioplegia in 24-hour preserved hearts. Ann Thorac Surg 1994;58:1651-6.
5. Milliken JC, Billingsley AM, Laks H. Modified reperfusate after long-term preservation of the heart. Ann Thorac Surg 1989;47:725-8.
6. Szabo G, Fazekas L, Bährle S, et al. Endothelin-A and –B antagonists protect myocardial and endothelial function after ischemia/reperfusion in a rat heart transplantation model. Cardiovasc Res 1998;39:683-90.
7. Boyle EM, Pohman TH, Cornejo CJ, Verrier ED. Endothelial cell injury in cardiovascular surgery: ischemia-reperfusion. Ann Thorac Surg 1996;62:1868-75.
8. Murphy CO, Pan-Chih, Gott JP. Guyton RA. Coronary microvascular reactivity after ischemic cold storage and reperfusion. Ann Thorac Surg 1997;63:20-7.
9. Morishita R, Sugimoto T, Aoki M, et al. In vivo transfection of cis element "decoy" against nuclear factor-kB binding site prevents myocardial infarction. Nat Med 1997;3:894-9.
10. Sawa Y, Morishita R, Suzuki K, et al. A novel strategy for myocardial protection using in vivo transfection of cis element 'decoy' against NFkappaB binding site: evidence for a role of NFkappaB in ischemia-reperfusion injury. Circulation 1997 Nov 4;96(Suppl):II280-5.
11. Neish AS, Williams AJ, Palmer HJ, Whitley MZ, Collins T. Functional analysis of the human vascular cell adhesion molecule 1 promoter. J Exp Med 1992:176;1583-93.
12. Kaneda Y. Virus(Sendai virus envelopes)-mediated gene transfer. In Cell Biology, a Laboratory Handbook. J.E. Celis, editor. Academic Press, New York 1994;Vol III:50-7.
13. Morishita R, Gibbons GH, Ellison KH, et al. Single intraluminal delivery of antisense cdc 2 kinase oligonucleotides results in chronic inhibition of neointimal hyperplasia. Proc Natl Acad Sci USA 1993;90:8474-8.
14. Sawa Y, Kaneda Y, Bai HZ, et al. Efficient transfer of oligonucleotides and plasmid DNA into the whole heart thorough the coronary artery. Gene Therapy 1998;5:1472-80.
15. Ono K, Lindsey E. Improved technique of heart transplantation in rats. J Thorac Cardiovasc Surg 1969;57:225-9.
16. Baxter K, Howden BO, Jin X, Jablonski P. Studies in a modified auxiliary abdominal rat heart transplantation model: preservation with colloid-free University of Wisconsin solution. J Heart Transplant 1998;17:532-7.
17. Gojo S, Niwaya K, Taniguchi S, Nishizaki K, Kitamura S. Gene transfer into the donor heart during cold preservation for heart transplantation. Ann Thorac Surg 1998:65:647-52.
18. Mann MJ, Gibbons GH, Hutchinson H, et al. Pressure-mediated oligonucleotide transfection of rat and human cardiovascular tissues. Proc Natl Acad Sci U S A 1999;96:6411-6.
19. Oz MC, Liao H, Naka Y, et al. Ischemia-induced interleukin-8 release after human heart transplantation. A potential role for endothelial cells. Circulation 1995;92(Suppl):II428-32.
20. Haddix TL, Pohlman TH, Noel RF, Sato TT, Boyle EM, Verrier ED. Hypothermia inhibits human E-selectin transcription. J Surg Res 1996;64:176-83.

Table –1. Hemodynamic parameters of the recipient

Group	HR(beats/min)	MAP (mmHg)	RT (°C)	Intusion (ml)
NF (n = 6)	431 ± 18	72 ± 5	35.9 ± 0.2	2.5 ± 0.3
SD (n = 6)	430 ± 12	68 ± 9	36.5 ± 0.6	2.5 ± 0.3

Data are expressed as mean ± S.E.M. There was no significant difference in any parameter between two groups. N = number of samples; HR = heart rate; MAP = mean arterial pressure; RT = rectal temperature.

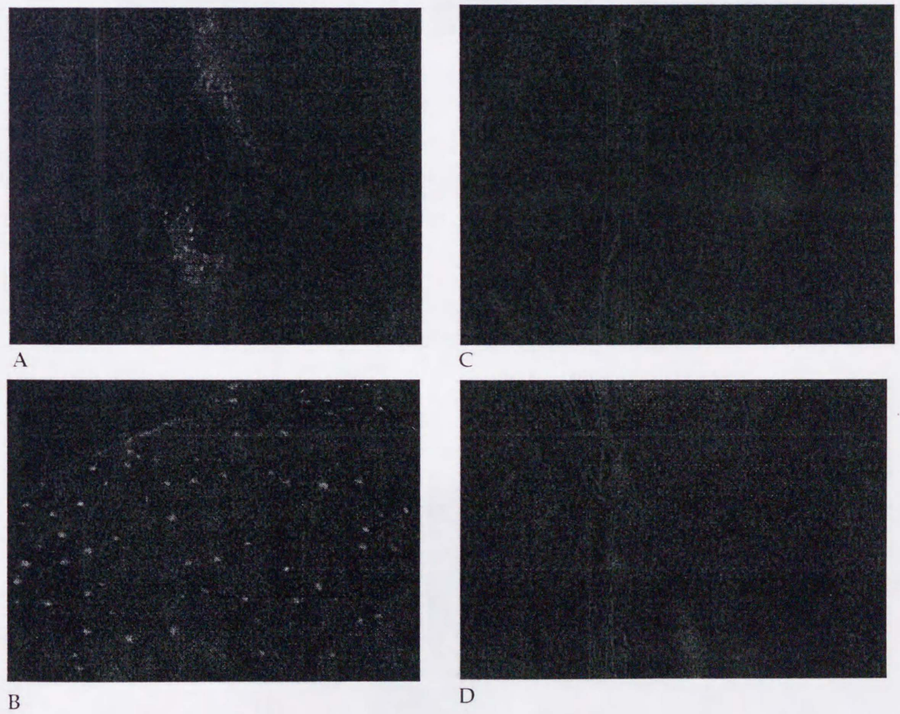

Fig 1. Ex-vivo distribution of FITC-labeled ODN delivered via infusion into a coronary artery by HVJ-liposome method (A,B) or direct transfer of FITC-labeled ODN (C,D). With the aid of the HVJ-liposome, vessels are preferentially transfected after 16-hour preservation (A, X 400), and cardiomyocytes are also transfected after subsequent reperfusion (B, X 100). No apparent staining is noticed with direct transfer both after preservation (C, X 400) and after following reperfusion (D, X 100).

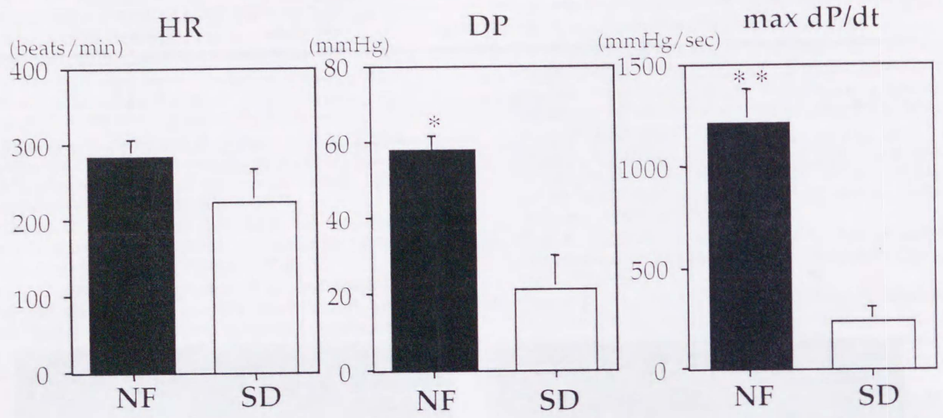

*Fig 2. The enhancement of cardiac preservation in terms of the recovery of cardiac performance after 1 hour of reperfusion by transfection of NFKB decoy. The NF group showed significantly higher recovery of left ventricular developed pressure (DP) and maximal derivatives of left ventricular pressure (max dP/dt) than the SD group, whereas there is no significant difference in heart rate (HR) between two groups. * P < 0.05, ** P < 0.01, n = 6 in each group. All values are expressed as mean ± SEM.*

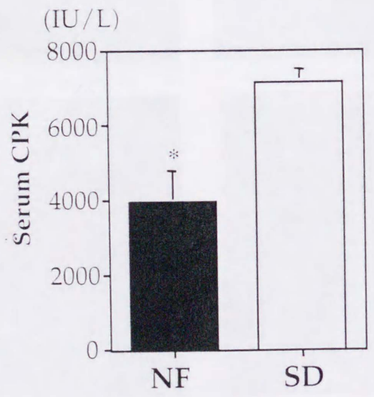

*Fig 3. Serum value of creatine phosphokinase (CPK) after 1 hour of reperfusion. The NF group showed significantly higher value of serum CPK than the SD group. * P < 0.05, n = 6 in each group. All values are expressed as mean ± SEM.*

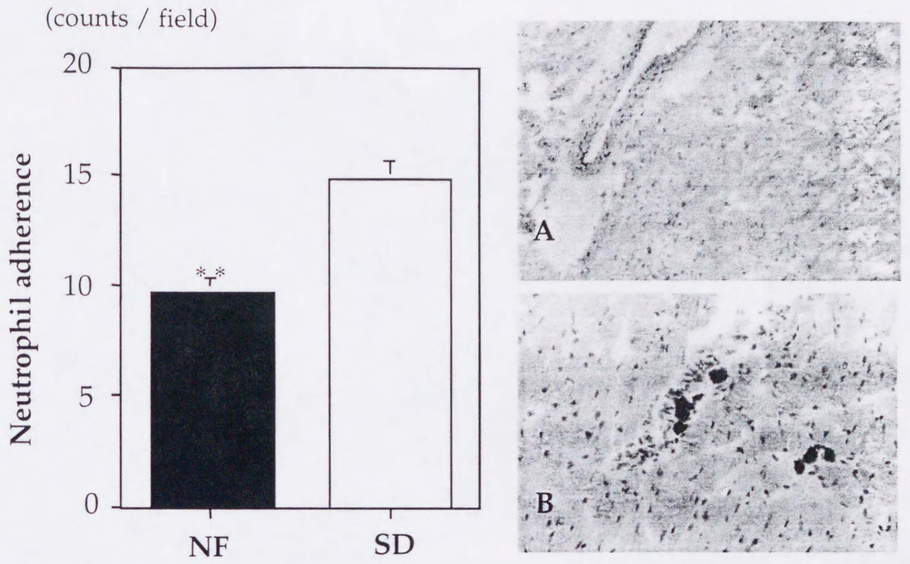

*Fig 4. Cytohistochemical staining for neutrophil adherence after reperfusion in the NF group (A; X 200) and in the SD group (B; X 200). The number of neutrophils is significantly smaller in the NF group than in the SD group. ** P < 0.01, n = 6 in each group. All values are expressed as mean ± SEM.*

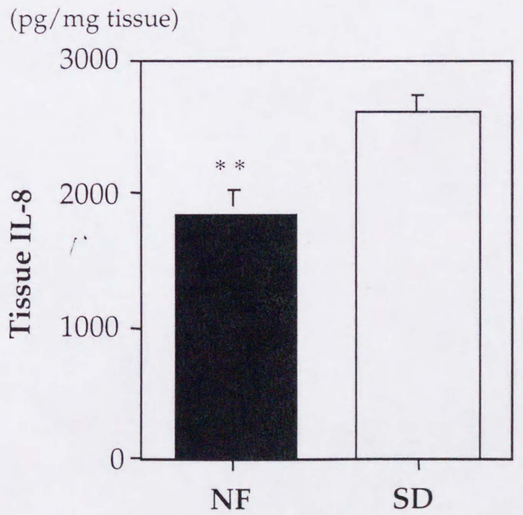

*Fig 5. Tissue IL-8 level measured by ELISA is significantly lower in the NF group than in the SD group. ** P < 0.01, n = 6 in each group. All values are expressed as mean ± SEM.*