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Experimental Study on Reperfusion Injury to Warm Ischemic Lung

Protective effect of CV-3611 and α-tocopherol on reperfusion lung

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ABSTRACT: In the experiment of a donor lung with a three hour warm ischemia the effects of antioxidant drugs, CV-3611 and α-tocopherol, on reperfusion injury were evaluated on dogs with left lung transplantation.

1) CV-3611 and α-tocopherol are of great benefit to prevent a donor lung from reperfusion injury, remaining satisfactory alveolar ventilation.
2) The addition of CV-3611 and α-tocopherol was depressed enough to generate the volume of lipid peroxidase in the lung tissues.
3) The pulmonary vascular resistance increased without exception in all groups, demonstrating no effect of antioxidant drugs.
4) The statistic compliance was remained satisfactory by α-tocopherol.
5) There was a tendency toward a decrease in oxidative product in neutrophils by CV-3611. This results suggest that CV-3611 and α-tocopherol should be of great use to avoid occurring reperfusion injury to ischemic lungs.

INTRODUCTION

Great concern regarding the problems to solve in a lung graft immediately after lung transplantation is to minimize reperfusion injury, which corresponds to acute lung edema. In 1985 McCord corroborated the concept of tissue injury caused by free radical at the time of reperfusion for ischemic myocardium and intestine and it is reported that the main cause of tissue injury is superoxide which generates from the ground substance of xanthine and hypoxantine (Fig. 1).

Recent study clarified that one of the cause of reperfusion lung injury is peroxidation product generated by granulocytes$^{2,3)}$. This study aimed at improvement of a result of organ transplantation by making use of protective effects of α-tocopherol and CV-3611, derivates of vitamin C, to ischemic preserved lung.

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*Fig. 1. Proposed Mechanism for Ischemia-Induced Production of Superoxide.*
MATERIALS AND METHODS

1) Experimental animal
Mongrel dogs weighing from 8 to 13 kg were used in this study. These dogs were supplied from the Animal Center of Nagasaki University School of Medicine.

2) Method
Mongrel dogs were anesthetized and maintained on sodium pentobarbital, intubated with a cuffed endotracheal tube and ventilated with 100% oxygen using a volume respirator (Harvard). The tidal volume set up to were 30 ml/kg, respiratory rate 12-14/min. A 5F Swan-Ganz catheter was inserted from the external jugular vein.

Thoracotomy was made at the 5th ICS on the left side and hilar stripping was performed by mobilizing the bronchus, pulmonary artery and vein and also dividing the bronchial artery, lymphatic channel and vagal nerve branches.

After administering heparin 200 U/kg intravenously, left pulmonary function was evaluated by temporary blocking test of the right bronchus and pulmonary artery.

Cardiac output and the pressure of the pulmonary artery were measured and static lung compliance was calculated by airway pressure-volume curve.

Warm ischemic preservation for three hours was continued on the condition of 60 to 80% inflated state by room air and dogs were divided into the three groups.

Group 1: simple warm ischemia (N=6).
Group 2: CV-3611 20 mg/kg per os administration one hour prior to induction of anesthesia (N=7).
Group 3: a-tocopherol 20 mg/kg iv administration immediately after thoracotomy (N=7).

Unilateral occlusion tests of right pulmonary artery and bronchus by the same way as made prior to ischemia were repeated at 10, 60, 90 and 120 min following reperfusion.

At the same time, generation of superoxide in granulocytes of the pulmonary vein and lipid peroxide in serum and lung tissues were measured. Histologic examination of lung tissue was made by H-E staining 120 min after reperfusion.

1) Measurement of lipid peroxide in blood and lung tissue
Fluorescence method was used for measuring lipid peroxide as reported by Yagi and the values were corrected by containing protein volume.

(1) Serum
Two ml of heparinized blood were centrifuged at 3000 rpm for 10 min, added 4 ml of 1/12N H₂SO₄ and 0.5 ml of 10% phospho-tungसic acid at room temperature for 5 min, centrifuged at 3000 rpm for 10 min. Added 2 ml of 1/12N H₂SO₄ and 0.3 ml of 10% phospho-tung스ic acid centrifuged at 3000 rpm for 10 min, added 4 ml of distilled water and added 1 ml of TBA reagent, heated at 95°C for 60 min, cooled at ice water bath, and vibrated with 5 ml of n-butyl alcohol, centrifuged at 3000 rpm for 10 min and measured by excitation spectrum (515 nm) with 553 nm fluorescence.

2) Lung tissue
After taking a piece of lung tissue, it was stored in -160°C liquid nitrogen, adding nine times volume of water and adjusted to 10% homogenized liquor and 0.2 ml 8.1% sodium dodecyl sulfate was mixed with 1.5 ml of 20% acetic acid buffer (pH3.5), 1.5 ml of 0.8% TBA-reagent and 0.6 ml of distilled water, heated at 95°C for 60 min, cooled at ice-bath and mixed 1 ml of distilled water with 15:1 admixture of n-butyl alcohol and pyridine, centrifuged at 3000 rpm for 10 min, and measured by butyl alcohol layer by excitation spectrum (515 nm) with 553 nm fluorescence.

3) Protein measurement
Serum was diluted 500 times with distilled water, finally homogenized liquor of lung tissues was diluted 1000 times. Three ml of Lowry C solution was added to 0.6 ml of homogenized liquor of serum and lung tissue, incubated at 25°C for 10 min, added 0.3 ml of 50% phenol solution and incubated at 25°C for 30 min, and made spectrophotometric measurement with 750 nm and calculated as the following equation:

serum: \[ \text{TBA} = \frac{f}{F} \times 0.5 \times \frac{1}{0.02} \text{nmol/ml} \]

correction by protein

lung tissue: \[ \text{TBA} = \frac{f}{F} \times \frac{1}{0.2} \text{nmol/ml} \]

\( f \): fluorescence of sample
\( F \): fluorescence of standard
\( Y \): 500 times diluted protein

lung tissue: \[ \text{TBA} = \frac{f}{F} \times 1/0.2 \text{nmol/ml} \]
correction by protein
TBA = F/F X 1/0.2 ÷ Y(100/1000) nmol/mg protein
Lipid peroxidase value of serum immediately before reperfusion was regarded as 1 and that of lung tissue was used as the measured value.

2) Measurement of oxidative product by neutrophils

Oxidative product by neutrophils was measured according to Flowcytometry method by Bass and modified by Taniguchi.

0.1ml of heparinized blood was added 1.9ml of 5μM 2', 7'-dichlorofluorescin diacetate (DCFHDA) and 0.5ml of EDTA which was adjusted to 24mM with PBS to avoid agglutinating neutrophils and added 10μl of phorbol myristate acetate (PMA), incubated at 37°C for 25min, washed and centrifuged at 1300rpm for 10min, treated with 0.87% NH₄Cl lysing solution, rewashed with PBS and centrifuged at 1300rpm for 10min, suspended pellet with Hanks’ solution and measured with Spectrum III (Ortho Co.).

B%start regarded the resting state in peripheral blood of dogs as 2-3% was set as negative control and B%regarded as generation capacity after PHA stimulation.

Figure 3 showed cytogram, indicating sizes of cells in foward light scatter of abscissa and internal structure of cells in 90 degree light scatter of ordinate.
Figure 4a showed a histogram by SPIII at resting state and Figure 4b also showed a histogram at PHA stimulation, indicating a formal distribution of neutrophils by stimulation.

The results were compared as an indicator of generation capacity of oxydative product (B%/X Bmch) with the control value (1) immediately before reperfusion.

3) Intrapulmonary shunt ratio

Intrapulmonary shunt ratio was calculated by simplified equation by Osaki:

\[ Q_s/Q_t = 0.003 \times \frac{AaDO_2}{4.5 + AaDO_2} \]

\[ PAO_2 = 1.0 \times (760 - 47 - PaCO_2) \]

\[ AaDO_2 = 713 - PaCO_2 - PaO_2 \]

4) Pulmonary vascular resistance (PVR)
PVR was calculated according to the following equation

\[ 80 \times \frac{PAP - LAP}{CO} \text{ (dyne-sec/cm}^2) \]

LAP: left atrial pressure
CO: cardiac output

5) static pulmonary compliance (ml/cmH2O)
The intratracheal pressure, flow speed, and tidal volume were simultaneously delineated on the graph, and the static pulmonary compliance was calculated by dividing tidal volume by intratracheal pressure 1.4 sec after end-inspiration, holding inspiration.

6) Pulmonary extravascular water (PEVW)

PEVW was calculated according to the following equation

\[ W - D/W \]

W: wet lung weight
D: dry lung weight

CV-3611 was supplied from Takeda Pharma Co and α-tocopherol from Eizai Pharma. Co. Data were expressed as means±SD.

Statistical significance for the data was estimated by Mann-Whitney U-test and P-values of less than 0.05 were considered to be significant.

RESULT

1) PaO2 (Fig. 5a)

PaO2 in Group 1 decreased in 271.6±111.5mmHg 10min after reperfusion in contrast to 429.5±79.6mmHg prior to reperfusion and continued to a decrease.

In Group 3 PaO2 somewhat decreased although those in Group 2 and 3 did not reduce afterwards. PaO2 60min later in Group 2 and 3 (375.9±70.6 and 355.5±83.0) remained significantly high (p<0.01) as compared with those in Group 1 (213.0±59.1) and an excellent oxygenation was shown.

(2) PaCO2 (Fig. 5b).

In Group 1, PaCO2 (38.5±9.3mmHg) was increased 60min after reperfusion as compared with that (27.4±4.3) prior to reperfusion. Meanwhile those in Group 2 and 3 did not increase, showing a significant reduction (p<0.05) in Group 3 immediately after reperfusion and in Group 2 90 min after reperfusion. It was a reflection of satisfactory alveolar ventilation.

(3) Intrapulmonary shunt ratio (Fig. 6)

In Group 1, intrapulmonary shunt ratio was
increased with time since 10 min after reperfusion, showing 21.1±4.4 10 min after reperfusion and later indicating a continuous increase. On the other hand, those in Group 2 and 3 did not increase although that in Group 3 showed a slight increase. It was statistical significance (p<0.01) between Group 1 (23.5±2.0) and Group 2, 3 (17.0±3.1, 17.9±3.7) 60 min after reperfusion.

(4) Pulmonary vascular resistence (Fig. 7)
PVR 10 min after reperfusion increased to 2433±607 dyne.sec.cm⁻⁵ as compared with that (1409±239) prior to reperfusion in Group 1 and also it was a similar increase in Group 2 (from 1245±518 to 2207±1041) and Group 3 (from 1282±389 to 2013±346). Thereafter, PVR remained high, showing a remarkable increase in Group 1 as compared with those in Group 2 and 3.

(5) Static lung compliance (Fig. 8)
Static lung compliance 120 min after reperfusion was reduced in all groups as compared with prior to reperfusion, from 31.3±4.7ml/cmH₂O to 21.9±3.7 in Group 1, from 29.2±3.7 to 25.6±6.1 in Group 2 and from 31.2±3.9 to 27.0±3.0 in Group 3. Those in Group 3 were significantly higher (p<0.05) than those in Group 1, showing a high tendency in Group 2.

(6) Oxidative product by neutrophils in blood of the pulmonary vein (Fig. 9).
In Group 1, oxidative product increased with time, 1.03±0.06 times 10 min after, 1.29±0.37 times 60min after and 1.39±0.32 times 120 min after reperfusion, as compared with that prior to reperfusion. In Group 3, it was almost the same tendency as 1.09±0.17, 1.33±0.39, 1.42±0.55. Meanwhile, in Group 2, it was depressed without statistically significant difference.

(7) Lipid peroxidase in blood of the pulmonary vein (Fig. 10).
In Group 1, lipid peroxidase content increas-
Fig. 10. Lipid peroxidase in serum

![Graph showing lipid peroxidase in serum over time.](image)

Fig. 11. Lipid peroxidase in lung tissue

![Graph showing lipid peroxidase in lung tissue comparison.](image)

Fig. 12. Pulmonary extravascular water

![Graph showing pulmonary extravascular water levels.](image)

ed with time, without statistical significance, 1.062±0.095 10 min after, 1.123±0.198 60 min after and 1.197±0.139 120 min after reperfusion as compared that prior to reperfusion. There was no any variation between those in Group 2 and 3 and no difference between Group 1 and 2 3. (8) Lipid peroxidase in lung tissues (Fig. 11)

Lipid peroxidase content in lung tissues 120min after reperfusion showed 0.939±0.112 nmol/mg protein in Group 1, 0.741±0.088 in Group 2 and 0.652±0.074 in Group 3. It was a remarkable reduction (p<0.01) in Group 2 and 3.

(9) Pulmonary extravascular water (PEVW)

PEVW 120 min after reperfusion showed 87.39±0.97% in Group 1, 86.53±1.61 in Group 2 and 85.37±2.44 in Group 3. These were a slight decrease without significant difference in Group 2 and 3 as compared with that in Group 1.

(10) Histologic examination

In Group 1, there was a significant finding of alveolar edema, destruction of alveolar structure and perivascular bleeding. On the other hand, in Group 2 and 3, alveolar structure remained almost normal in spite of a presence of edema in the alveolar septum with the slight degree of perivascular bleeding.

DISCUSSION

Many previous reports have shown that the maximum tolerable time duration of experimental animals lies between 30 and 60 min in collapsed lungs at room temperature, two hours in ventilated lungs three hours in continuous inflated lungs. The major item of concern about lung storage is how to prevent a stored lung from reperfusion injury (RPI). Many research work focus on the causes of reperfusion injury which is provoked by activation of xanthine oxidase system and generation of hydroxyl radical from activated neutrophils. Recently it is defined that human heart muscles do not contain XOD despite containing large amounts of XOD in the human lung tissues. The debate about generation of hydroxyl radical in XOD system are centered around the genesis that hypoxanthine originated from ATP during the ischemic phase reacts activated SOD, resulting in generation of O2·− via H2O2. On the consequence, ·OH is generated and causes severe damage to the cells. The mechanisms of cell damage caused by neutrophils are complex. Keith reported that tissue damage is caused by a large amount of hydroxyl radical which is released by neutrophils activated by C5a and complement. The other hand, it is said that activated neutrophils release elastase which is consequent to inactivation of α1-antiprotease by
reacting with oxidative product\(^2\).

Furthermore, oxidative product induces an inhibition of Ca\(^{2+}\) transport in the endoplasmic reticulum of muscles\(^7\). As a result, Ca\(^{2+}\) makes phospholipase active and arachidonic acid cascade reaction progressive which results in leukotriens generation\(^18\).

It is well known that LTB\(_4\) stimulates neutrophils and leukotxin generation is accelerated, forming a vicious circle of cell damage. Some researchers reported that a ultra-long acting HOCL is generated from myeloperoxidase originated from neutrophils\(^19\).

It is accepted that in all instances, oxidative product may trigger damage to tissue, therefore antioxidative drugs are necessary for avoiding and alleviating reperfusion injury syndrome. Meanwhile, it is well known that ascorbin acid is capable for inactivation of O\(_2^-\) which generated in water-soluble fraction such as the cell membran and also acts as an excellent scavenger for •OH in the neutral status\(^20, 21\). In contrast, in a presence of free metal of Fe\(^2+\) and Cu\(^{2+}\) and so on, ascorbin acid accelerate generation of oxidative product which is so-called prooxidant action. A drug of CV-3611 is fat-soluble ascorbin acid, unlikely to be affected by free metal ion in cells and poseing only action antioxidat agents.

Many studies clarified that CV-3611 inhibits generation of lipid peroxidase in accordance with its concentration\(^2\) and also prevent ischemic heart from reperfusion injury\(^23\). At present, as the titers of CV-3611 are reduced by dissolution following oral admistration, so every effort has been made to minimize it in a very few reports. Hashimoto\(^24\) reported that it continues to act during a six hours duration. In this series, 20mg/kg CV-3611 were orally administered 60 min before induciton of anesthesia since it takes six hours after reperfusion. There was a tendency toward reduction in generation of oxidative product of neutrophils until 90 min after reperfusion in accordance with Kuzuya' report\(^2,3\). It is reasoned that CV-3611 plays a key role either in inhibiting Harbar-weiss reaction (O\(_2^-\)+H\(_2\)O\(_2\)->O\(_2\)+OH + •OH) by inactivation of O\(_2^-\) or in inhibiting Fenton reaction (H\(_2\)O\(_2\)+Fe\(^{2+}\)->Fe\(^{3+}\)+•OH+OH\(^-\)) by impaired conversion process of Fe\(^{3+}\) to Fe\(^{2+}\)

as a result of blocking of activation pathway\(^3\) of neutrophils with help of a presence of C\(_{5a}\). On the other hand, Halliwell et al\(^25\) reported that ascorbic acid biologically scavenges oxidant hypochlorous acid by inactivation of α-antiprotease. There are few reports regarding protection from reperfusion injury on the lung although some reports clarify that vitamine E (α-TOC) protects brain ischemia\(^26\), endotoxic damage\(^27\), reperfusion injury from renal ischemia\(^28\) and myocardial protection\(^29\). It is well known that α-TOC is present in the cell membrane and inhibits generation of lipid peroxidase by reacting with lipid peroxiradical and free radical whic is generated in the cell membrane\(^30, 31\), although it does not almost react with O\(_2^-\) generated via xanthin oxidase system. Reddanna\(^32\) reported that α-TOC inhibits 5-lipoxygenase activity and also prohibits generation of LT which acts as a strong tissue destruction. From a result of this study, it is not certain that α-TOC directly inhibits generation of oxidative product from neutrophis. The reason for activation of neutrophils is that α-TOC neither react with O\(_2^-\) nor inhibit C\(_{5a}\) production. It is suggested that the mechanism causing cell damage by leucocytes should be that activated leucocytes adhere to the endothelium of the vessel, and on the consequence, elastase released from activat-ed leucocytes causes damage to endothelium of the vessel, change in matrix beneath the basal membrane and progress in destruction of alveolar epithelium\(^2\).

From this study, it was certified that CV-3611 and α-TOC administrations functionally produced satisfactory alveolar ventilation, reflecting inhibited generation of lipid peroxidase in the lung. In contrast, both drugs could not be beneficial in preventing an increase in pulmonary vascular resistance and extravascular lung water.

Histologic examination revealed that alveolar structure was kept almost satisfactory in drugs administration as compared with that in non-drugs administration. However, the findings of perivaschlar edema and bleeding in drugs were almost the same as non-drugs administration.

It is concluded that CV-3611 and α-TOC are not so effective as to be expected to prevent a
donor lung from causing damage to the endothelium of the vessels. In contrast, these are of great benefit to prevent a donor lung from changes in matrix beneath the basal membrane and destruction of alveolar epithelium.

Some reports certified that α-TOC benefits the storage organs from elimination of lipid peroxidase generated by the cell membrane and it also facilitates antioxidative actions of ascorbic acid and α-TOC. This study clarified that static compliance used as a parameter of lung edema was remained satisfactory and generation of lipid peroxidase in the lung was depressed. These results in this series were consistent with those which were drawn by reports described previously.

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