行政院國家科學委員會補助專題研究計畫成果報告

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※ Adjunctive 17β-Estradiol Reduces Myocardial Infarc ※
※ Size in the Canine Heart of Regional Ischemia and ※
※ Reperfusion. ※
※ 使用17β雌二醇附加治療在實驗狗心臟局部缺血和 ※
※ 重灌注損傷中會減少心肌梗塞的範圍 ※
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計畫主持人：周迺寬

共同主持人：李聰明

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行政院國家科學委員會專題研究計畫成果報告

Adjunctive 17β-Estradiol Reduces Myocardial Infarct Size in the Canine Heart of Regional Ischemia and Reperfusion.

使用 17β雌二醇附加治療在實驗狗心臟局部缺血和重灌注損傷中會減少心肌梗塞的範圍

計畫編號：NSC 89-2314-B-002-288
執行期限：89 年 08 月 01 日至 90 年 07 月 31 日
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計畫參與人員：xxxxxx　執行機構及單位名稱

一、中文摘要

目的：氧化劑在心肌缺氧重灌注梗塞的病理生理機轉扮演重要角色，而動情激素有抗氧化作用，可以保護梗塞過的心肌。本實驗目的在狗實驗心肌缺氧模型，使用動情激素後，在重灌注心肌時的心肌保護程度研究。

方法：實驗狗被全身麻醉，將其冠狀動脈左前下降枝阻斷 60 分鐘，再重灌注兩小時，全部實驗使用十八隻狗，隨意地分成三組，第一組 6 隻狗為控制組；第二組 6 隻狗在冠狀動脈阻斷前 15 分鐘使用 17β雌二醇，每公斤 5 毫克給予；第三組 6 隻狗則在六十分鐘阻斷缺氧後，在重灌注前三分鐘投予 17β雌二醇，最後在重灌注兩小時後，將梗塞後的心肌在電腦輔助下，給予梗塞部分量化，而心肌切片染色為 methyl blue 及 triphenytetra zolium chloride，而使用超音波都卜勒血流測量導線置於阻斷冠狀動脈遠端測其側枝循環血流係數。

結果：在三組實驗中，其血型動力學變異及側枝循環血流係數在阻斷時期變化，在統計上沒有差異，但是三組中，第一組控制組其梗塞面積為 37±7%，但二組為 15±6%，第三組為 18±9%，其差異 P 值分別為 0.0001 及 0.002。缺氧後 60 分鐘再灌注會造成自由基明顯上升，而三組中，活化的 Thiobarbituric acid 濃度相同，而 Diene conjagate 在第一組比較高。

結論：在缺氧前或缺氧後重灌注前投與動情激素可以減少缺氧梗塞的心肌面積，可能動情激素可以控制自由基的上升而有心肌保護作用。

關鍵詞：動情激素，自由基。

□、計畫成果摘要:

Abstract

Objectives: Oxidative stress plays an important role in the pathophysiology of ischemia-reperfusion myocardial infarct. Estrogen has an antioxidant potential that may contribute to its protective effect on infarct size. The purpose of the present study was to assess the cardioprotective efficacy of adjunctive administration of estrogen prior to reperfusion after prolonged ischemia in the canine model.

Methods: Anaesthetized dogs were subjected to 60 min of left anterior descending coronary artery occlusion followed by 2 h of reperfusion. Experiments were performed in 18 dogs, randomly assigned to receive vehicle (n = 6, control group), or 5 mg/kg of 17β-estradiol 15 min before coronary occlusion (n = 6, early group) or 3 min before coronary reperfusion

following 60- min ischemia (n = 6, late group). Myocardial infarction was produced by occlusion of the left anterior descending artery for 60 minutes followed by 2 h of reperfusion. At the end of the reperfusion period, area at risk and infarct size were quantified by computerized planimetry of slices of myocardium previously masked with methyl blue and triphenytetrazolium chloride. An index for collateral flow was determined by positioning the Doppler flow wire in the collateral-dependent vessel distal to the ligation and measuring the flow velocity.

Results: Hemodynamic variables and collateral flow during ischemia were similar in the two groups. Infarct size was larger in control (37 ± 7 % of area at risk) than in supplemented (15 ± 6 % in early group; 18 ± 9% in late group , p = 0.0001, 0.002, respectively). Reperfusion after 60 minutes of ischemia causes a significant elevation in free radicals. Although the changes of concentrations of thiobarbituric acid-reactive material were similar among the 3 groups, diene conjugates were significantly higher in control compared with in the two supplemented groups. ***Conclusion:*** Estrogen administration prior to reperfusion as an adjunct significantly reduces infarct size as good as estrogen administrated before ischemia. The cardioprotective effect of estrogen may result from inhibition of free radical rise.

Keywords: Diene conjugates; Estrogen; Free radicals; Intracoronary Doppler flow wire; Reperfusion injury; Thiobarbituric acid-reactive material

□、計畫簡介（Introduction）

1. Introduction

During the past few years, myocardial reperfusion therapy, such as primary percutaneous transluminal coronary angioplasty or thrombolytic therapy, has been widely performed in the management of acute myocardial infarction. Although restoration of blood flow arrests the progression of necrosis, paradoxically it is accompanied by functional derangement, including the increase of infarct size. It has been well known that a burst of free radical formation releases followed by reperfusion after prolonged ischemia [1,2]. Oxygen-derived free radicals have been proposed as general mediators of tissue injury in a variety of disease states. Much evidence now indicates that oxygen free radicals have been implicated as a cause of deleterious effects in the setting of coronary reperfusion. Free radicals are thought to form lipid peroxides that deplete phospholipids from cell membrane, inhibit membrane–associated enzymes, and interacts with proteins to cause tissue damage [3,4]. Free radicals are thought to form lipid peroxides that deplete phospholipids from cell membrane, inhibit membrane–associated enzymes, and interacts with proteins to cause tissue damage [5,6]. Pretreatment with the oxygen radical scavenger superoxide dismutase had a protective effect, suggesting that oxygen radicals were the cause of this ultrastructural injury. Given their deleterious effects of cells, it is not surprising that reperfusion-related free radicals eventually extent the myocardial infarction. With the knowledge of deleterious effects of free radicals during reperfusion period, adjunctive treatment of reperfusion injury has been the focus of intense investigation.

Estrogen, especially estriol and 17β-estradiol, which possess a phenolic hydroxyl group, have an effective antioxidant action and inhibit lipid peroxidation [7]. Kim et al [8] showed that chronic administration of supraphysiological dose of estrogen as an antioxidant prevents dysfunction of myocardium damage after ischemia and reperfusion in canine models. In vitro studies have shown that supraphysiological levels of estrogen in micromolar concentrations effectively scavenge radicals [9,10]. However, in physiological levels of estrogen (picomolar) the antioxidant effect of estrogen has been controversial. Some studies [11,12] showed an inhibition of in vitro oxidation of LDL by physiological concentrations of estrogen. In another comparable study, the investigators failed to

demonstrate that estrogen could act as an antioxidant to inhibit LDL oxidation [13] Previous studies [14,15] on cardioprotection of estrogen have limited the timing of drug administration prior to coronary occlusion. Because treatment prior to acute myocardial infarction is a virtual impossibility in most clinical situation, there has been a great deal of interest in anti-infarct agents that do not require pretreatment (adjunctive treatment). The aims of the study was (1) to test whether estrogen in physiological concentrations can act as an antioxidant in ischemia/reperfusion model; (2) to assess the effects of adjunctive estrogen administrated just prior to coronary reperfusion on infarct size; (3) to evaluate the cardioprotective effect of estrogen was direct acting during the reperfusion period, or indirect, arising as a consequence of the effects of the drug on the heart during ischemia.

□、材料及方法(Subjects and Methods)

Preparation. All experiments were conducted on mongrel dogs of either sex, weighing 10-15 Kg. The dogs were intubated and will be mechanically ventilated (Bennett MA-2) by using room air, will deliver at a rate of 20-25 strokes per minutes and a tidal volume of 300 ml. Because of epicardial temperature is a major of myocardial infarct size [16], body temperature was maintained at $38 \pm 0.5^{0}C$ by means of a temperature probe thermometer inserted into the pericardial cradle so that its sensor surface was adjacent to the posterior surface of the heart. The thermometer was attached to a homeothermic blanket control unit (Gaymar Medi-Therm II). A standard limb-lead electrocardiogram was recorded. Arterial blood samples were taken periodically to determine Po_2, Pco_2, pH, oxygen saturation, hemoglobin concentration, sodium, potassium, chloride and calcium concentration. Sodium bicarbonate solution was administered intravenously to adjust the acid-base balance when necessary. Left ventricular cavity and systemic blood pressures were measured with catheter-tip micromanometers inserted via the left ventricular apex and right femoral artery, respectively. The reference pressure level that was set at the right atrial level and used for all pressure measurements. Hydration was maintained by a slow 2.5% glucose infusion. Because operation-related catecholamine release has been implicated in the mechanism of ischemic preconditioning [17], each dog will have the same time (15 minutes) for stabilization before the study. Then hemodynamic parameters, including heart rate, arterial blood pressure, and left ventricular end-systolic/end-diastolic pressure were continuously recorded on a 4-channel polygraph recorder (Hewlett-Packward model M1962A). Additional thiopental anesthesia was given during the study as required to maintain a deep level of anesthesia.

The chest was opened through the left fourth intercostal space, the pericardium was opened, and the heart was exposed. Near the base of the heart, the left anterior descending artery proximal to first diagonal branch will be encircled with a 4-0 silk suture. The two ends of the suture were threaded through a piece of tubing, forming a snare that could be tightened to occlude the artery. Coronary occlusion in this region usually results in a large ischemic area of the anterolateral and apical regions of the left ventricular wall. Myocardial ischemia was confirmed by regional cyanosis, acute electrocardiographic ST segment elevation, and coronary flow changes detected by intravascular Doppler flowire. Reperfusion was effected by releasing the snare and was confirmed by visible hyperemia over the surface and hyperemic Doppler shift.

To measure collateral blood flow at baseline and during ischemia, coronary blood flow was detected by intracoronary Doppler flowire (FloWire, Cardiometrics, Inc, Mountain View, CA), instead of the universally recognized microsphere techniques which were proved to be insensitive to detect collateral flow [18]. Digitized spectral peak velocity waveforms were averaged to compute the average peak velocity (APV) (cm/sec). The monitor

display was continuously recorded on Super VHS videotape for off-line analysis. To determine cross-section area of the artery, a 5-mm segment was measured immediately distal to the tip of the Doppler catheter. Volumetric coronary blood flow (CBF) was calculated as CBF (ml/min) = CSA (mm^2) xAPV (cm/sec) x0.5x0.6. as validated by Doucette et al [19], where CBF is coronary blood flow (ml/min), CSA is cross sectional area (mm^2), and APV is averaged peak velocity (cm/sec).

All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Subjects Committee of the National Taiwan University.

Experimental protocol. The dogs were assigned randomly to one of three groups. All animals were subjected to a 60-minute coronary occlusion followed by 120 minutes of reperfusion. *Group I (control)* as the control group and only vehicle (5 ml saline) was administrated prior to the 60-minute occlusion. In *Group II (early group)*, animals were treated with intravenous 10 *ug*/Kg of 17β-estradiol (dissolved in 1.0 ml ethanol, Sigma Chemical Co., St. Louis, MO.) prior to the 60-minute occlusion. In *Group III (late group)*, animals were treated with intravenous 10 *ug*/Kg of 17β-estradiol 3 minutes just prior to reperfusion after the 57-minute occlusion. The doses were chosen to achieve serum estradiol levels in the range of 200 to 500 pg/ml, levels that are equivalent to those in human females during midcycle.

At the end of the protocol, the coronary artery was clamped, and 4 ml of a solution of methylene blue dye (Sigma Chemical Co., St. Louis, MO.) was injected into the left ventricular apex, defining the ischemic region at risk of necrosis. The deeply anesthetized dog was then killed by an injection of hypertonic potassium chloride. The heart was sliced transversely into six to eight sections, and the slices were photographed to record the ischemic areas (uncolored by the blue dye) and the nonischemic, normal areas (perfused blue) in each slice. After a 10-minute incubation in a 1% solution of buffered triphenyltetrazolium chloride (TTC), the slices were again photographed to record the necrotic regions (unstained by TTC) and the noninfarcted regions (stained red by TTC). Later the photographic slides were projected and traced. The areas of ischemic and normally perfused regions and the areas of necrotic and nonnecrotic regions were measured on the tracings by computerized planimetry (Image Pro Plus, CA). Three areas were multiplied by the weight of each slice, and the results were summed to obtain the mass of risk and infarction.

To verify the reproducibility of the computer-assisted planimetry, a second measurement was performed by another blinded investigator. The correlation between the two measurements was excellent ($r = 0.99$ for ischemic area).

Histology analysis.

Extensive histological samples were taken from each transverse section, processed by conventional methods, and stained with hematoxylin and eosin, and Masson for contraction band. A pathologist (CCT) who was unaware of the treatment protocol examined the samples for microscopic evidence of contraction band necrosis on random fields at a magnification of 400X. The results from the section in each case with the highest number of bands was used. The histologic severity of contraction band necrosis was used to grade injury on a scale of 0 to 3 for the number of contraction bands: 0 (absent), 1 (mild), 2 (moderate) and 3 (severe) which represented 1, 1 to 5, 6 to 10, and 11 or more cells with contraction bands per sq mm of tissue, respectively [20]. Reproducibility of the method for grading contraction band necrosis was assessed by analyzing interobserver and intraobserver variabilities. Interobserver variability was determined by having a second observer (TML) reanalyze staining from the original preparations analyzed by the first observer. The calculated coefficients of interobserver and intraobserver variations were less than 9% and 5%, respectively.

Free radical assays. Serial serum samples for free radical measurements were withdrawn at baseline (15 minutes after stabilization), 15 minutes after 17β-estradiol

administration (early group as baseline), 15 minutes after coronary ligation, immediately before coronary reperfusion, 2, 5, 15, 60 minutes after reperfusion, and at the termination of reperfusion (2 hours). Samples were centifuged at 2000 *g* at 4^0C for 20 minutes. Plasma specimens were stored at -70^0C in plastic tubes until assay.

Thiobarbituric acid (TBA) reactive material (malondialdehyde). Plasma samples were dilated in phosphate buffer and heat together with a TBA solution (325 mg/ml) in a boiling water bath for 15 minutes [21]. The tubes were then collected and absorbances measured at 535 nm. The coefficient of variation between assays in our laboratory has been 12%.

Diene conjugates. Free radical damage to polyunsaturated fatty acids does not invariably lead to lipid peroxidation. Under certain conditions free radicals can cause isomerization of lipid acyl chains to yield diene conjugates without the addition of oxygen [22]. Such products will not be detected in the assay for TBA-reactive material. Lipids were extracted from the plasma samples by chloroform-methanol (2:1), dried under nitrogen, then redissolved in cyclohexane, and analyzed spectrophotometrially at 323 nm [23]. The coefficient of variation between assays in our laboratory has been 17%.

Laboratory measurements. 17β-estradiol concentrations were quantified by enzyme-linked immunoassay (Diagnostic Products Corporation, Los Angeles, CA) before and at the end of the study. The detection limit was 20 pg/ml for estradiol.

Exclusion criteria. Animals were omitted from analysis: (1) if such severe hypotension was observed that the experiment could not be continued successfully for the duration of the protocol; (2) if intractable ventricular fibrillation occurred or arrhythmia needed antiarrhythmic agents to correct; (3) collateral flow > 20% of baseline coronary blood flow; or (4) presence of heart worms. Dogs with ventricular fibrillation during reperfusion were resuscitated and converted to a stable rhythm by internal electric shocks (3x10 W + 1x25 W). The low energy did not result in more cell necrosis [24]. We calculated survival percentage as Number of Dogs that survived/Number of Assigned Dogs x100.

Statistics

We tested for the differences for hemodynamic variables, infarct size and area at risk among the three groups by one-way analysis of variance followed by a Newman-Keuls post hoc test. The chi-square test and Fisher's exact test were used to compare the frequency of survival animals after different treatments. Changes along time were assessed by multiple ANOVA analysis. For the comparisons of the severity of contraction band necrosis Gehan's generalized Wilcoxon test was applied. Linear regression analysis was used to determined the relationship between area at risk and infarct size as a percent of the left ventricle. A value of $P < 0.05$ was considered to be significant.

□、結果(Results)

Intravenous injection of 10 *ug*/kg of 17β-estradiol, either prior to coronary occlusion or prior to reperfusion, resulted in increases of plasma estradiol concentration (Table 1) that are equivalent to those in human females during midcycle. Concentration of arterial blood gas, calcium, sodium and potassium were fairly stable throughout the study.

3. 1. Mortality and exclusions.

A total of 28 animals was enrolled in the study. Seven animals in the control group were excluded, 3 because of collaterals, 3 for intractable ventricular fibrillation during coronary occlusion, and one for hypotension. Two animals were excluded in the early group, 1 for collaterals, and one for hypotension. One animal was excluded in the late group for collaterals. Significantly more animals survived during ischemia and reperfusion in the estrogen-treated (6/7, 86%, in the early group; 6/6, 100%, in the late group)

compared with the control (6/10, 60%; $P < 0.05$, both, respectively). The remaining dogs were randomly assigned to each group of 6.

3. 2. Hemodynamic Variables.

The hemodynamic data are summarized in Table 1. There were no significant differences in heart rate, mean aortic pressure, left ventricular end-diastolic pressure, and rate-pressure product. Blood flow of the anterior descending artery among the groups were similar throughout the experiment, suggesting that collateral flow to the ischemic region was not altered by estrogen treatment. The pressure rate index, an index of myocardial oxygen demand, was similar for the 3 groups throughout the study.

3. 3. Coronary blood flow.

Baseline coronary blood flow measured with intracoronary Doppler flow wire was nonsignificantly different among the 3 groups (Table 1). Five minutes after occlusion, collateral blood flow in the center of the ischemic region was very low in both groups, and increased only slightly with time. The amount of decrease after coronary occlusion was within the same range among the 3 groups. At the end of occlusion, there was no significant difference of collateral blood flow among the 3 groups.

3. 4. Free radicals.

During the period of cardiac ischemia there was no significant changes in either TBA-reactive material or in diene conjugates. During the first minutes after reperfusion, however, there were significant changes: the levels of diene conjugates increased significantly in control compared with those in early and late groups. The levels of TBA-reactive material were similar in the 2 groups throughout the experiment.

3. 5. Infarct size.

There were no differences in body weight, heart weight, or areas at risk (AAR) among the groups. There was no significant difference in AAR expressed as a percentage of left ventricle among the 3 groups, indicating that a comparable degree of ischemic risk. After 1 hour of coronary artery occlusion followed by 2 hours of reperfusion, the necrotic area, expressed as a percentage of the AAR or a percentage of left ventricle, was 37 ± 7% and 19 ± 4% in the control, 15 ± 6% and 7 ± 3% ($P < 0.0001$, both, vs. control) in the early group, and 18 ± 9% and 9 ± 4% in the late group ($P < 0.0001$,both, vs. control).

The correlation coefficient between infarct size and area at risk was 0.72 ($P < 0.0001$) in the control animals. 17β-estradiol treatment resulted in a significant downward shift in the regression line, with all infarct sizes smaller than predicted from the area at risk. The slopes of the regression lines in control and treated groups were statistically different (P = 0.001).

3. 6. Histological analysis.

Macroscopically, control dogs exhibited confluent infarctions, whereas infarcts in the early and late estrogen-treated groups were patchily distributed, interspersing with islands of viable myocardium. Histological analysis in all groups revealed infarcts composed almost exclusively of contraction band necrosis. The severity of contraction band necrosis was significantly higher in controls compared with the treated group. There was a good correlation between the extent of infarct size normalized by AAR and the severity of contraction band necrosis (r = 0.90, $P < 0.0001$).

□、討論(Discussion)

Our present results clearly showed for the first time that despite the late administration of estrogen prior to coronary reperfusion, the drug effectively reduced infarct size after myocardial ischemia. Estrogen was as effective when it was given just prior to reperfusion as it was when present throughout ischemia and reperfusion, which is

different from what previous studies have found with other preconditioning mimetics such as adenosine [25]. Thus, the infarct-limited effect of estrogen is through direct protection during coronary reperfusion. Such beneficial effects of estrogen in physiological concentrations appear to be mediated, at least in part, through the antioxidant activity of estrogen.

4. 1. Coronary blood flow.

Interpretation of our current results should take into account methodological considerations. Because the perfusion to ischemic zone is the determinant of whether ischemic myocardium survives or dies following acute occlusion of a coronary artery, numerous techniques have been developed for the measurement of coronary blood flow, including timed venous collection, thermodilution, labeled microspheres, colored microspheres, diffusible indicators, clearance methods, electromagnetic flow, and positron emission tomography [26]. Although radiolabeled microsphere method has merged as the technique of choice, it may seriously underestimate the total nutritional component of flow to an ischemic zone [18]. The microsphere-insensitive component could be as much as one-fourth of the total ischemic zone perfusion, which would represent a significant error in microsphere estimate [18]. Lack of dependence on pattern of nutritional flow makes the Doppler catheter technique better suited for assessing coronary collaterals during occlusion. Angiographically determined cross section area has a high correlation with intravascular ultrasound computed cross section area [27]. Therefore, previous studies [27,28] have validated the combined use of angiographically-derived cross section area and intracoronary Doppler velocity to measure the real-time coronary blood flow.

Reduction of the infarct size in the treated group was not explained by differences of collaterals. A major problem with the use of a canine model of regional ischemia for the study of ischemia-reperfusion is the presence of a significant collateral circulation that protects the ventricles from infarct and fatal arrhythmias at different degrees [29]. To make collateral circulation of our study dogs homogenous, we excluded dogs with collateral blood > 20% of baseline. Previous studies [30] showed only minimal changes in myocardial blood flow during coronary occlusion, which was consistent with our findings. In the present study, collateral blood flows during coronary occlusion among the 3 groups were similar, demonstrating that differences in infarct sizes were not explained by collateral blood flow. This finding was consistent with previous studies [31,32], demonstrating that estradiol-induced cardioprotection was independent of changes in regional myocardial blood flow.

4. 2. Mechanisms of Cardioprotecive Effects of 17β-estradiol

Estrogen in physiological concentrations exerts its cardioprotective effects by antioxidant activity assessed by diene conjugates. It is known that major determinants of myocardial infarct size are duration of coronary occlusion, size of the ischemia risk zone, amount of collateral blood flow in the ischemic zone, and hemodynamic determinants of myocardial oxygen demand (rate-pressure product) [30]. However, the above mechanisms appear not to be the mechanism of cardioprotective effect of 17β-estradiol. Kim et al [8] have demonstrated that the chronic administration of 17β-estradiol reduced infarct size through the antioxidant activity of estrogen. Antioxidants can exert their cardioprotective effects by reducing production of contraction band necrosis. Some myocytes which are reversibly injured at the time that coronary artery occlusion is terminated undergo irreversible cell damage during reperfusion [33]. Tissue areas that can be salvaged by agents after acute myocardial infarction may be areas with contraction band necrosis. Calcium overloading during reperfusion was supposed to play an important role in the formation of contraction band necrosis [34]. Free radical scavengers inhibit a marked increase in cytosolic calcium concentrations on reperfusion of ischemic myocardium [35]. Widman et al [36] demonstrated that free radical scavenger can modify the severity of

contraction band necrosis, which was consistent with our finding. Further, estradiol directly inhibits cardiac Ca^{2+} current [37] which could lead to a decreased influx of calcium concentration during reperfusion. Besides, estradiol may protect against apoptotic cell death induced by the free radicals. Cardiomyocyte apoptosis is the major form of early myocyte cell death (within 2 hours after reperfusion) produced by occlusion of a major epicardial coronary artery [38].

Herve et al [39] have demonstrated that 17β-estradiol reduced the gap junction communication in cardiac myocytes, which will reduce the formation of contraction band necrosis and eventually improve infarct size, consistent with our finding. Previous studies have demonstrated that traffic of potentially harmful cytosolic messengers between ischemic cells and surrounding nonischemic cells might cause amplification of injury [40], leading to myofibrillar hypercontracture and further precipitating cell death [41]. Cell-to-cell interactions via intercellular gap junction may damage adjacent myocytes. Gap junction uncouplers [42,43] have been reported to exert cardioprotective effects in ischemia/reperfusion models by blocking the cell-to-cell interaction. Whether such peculiar configuration of these infarcts suggested interfering with cell-to-cell progression of myocardial necrosis remained unknown. Our study was not designed to elucidate the underlying mechanisms.

The serum concentrations of diene conjugates were higher in the treated group than in the control group, but, in contrast to the TBA, they were not significantly different among the 3 groups. This discrepancy between the two indicators of free radical activity may simply be related to detect different products. In most of the studies a single index of free radical activity was measured. The use of two independent measures of free radical activity, as in the present study, has some theoretic advantages. Free radicals can cause the isomerization of lipid acyl chains, generating products with diene conjugation but without peroxidation. These products will not be detected by an assay for TBA. The different measures for free radical activity with different sensitivity and specificity could account for part of discrepancy between the results of the antioxidant role of estrogen in physiological concentrations and between results of peak timing of free radicals after reperfusion. Lindower et al [44] have demonstrated that myocardial generation of ascorbate free radicals was maximal 10 minutes after reperfusion by using an electron paramagnetic resonance, in contrast to our findings.

There have been conflicting data with regard to the benefit of free radicals scavengers: some studies [45,46] have shown infarct size reduction, whereas others [47] have not demonstrated myocardial salvage. The discrepancies could be due to differences in the animals models used, time of reperfusion, and histochemical methods [48]. Besides, because free radical scavengers may delay, rather than prevent, infarction, the duration of a plasma half-life could play e role to affect the infarct size. Superoxide dismutase has a plasma half-life of only about 10 minutes. Thus, it could disappear during much of the reperfusion phase. Tamura et al [49] showed that superoxide dismutase conjugated to polyethylene glycol, thereby extending the half-life to 30 hours, significantly and consistently reduced infarct size. Contrast to short action of unconjugated superoxide dismutase, the plasma half-life of 17β-estradiol is about 3 hours [50]. It could be expected that estrogen may provide a cardioprotective effect by its long elimination half-life.

4. 3. Other mechanisms.

Although, the present results suggest that antioxidant effects of estrogen contribute to the alleviation of reperfusion injury, we must carefully consider other possible mechanisms for the cardioprotecive effects of 17β-estradiol. First, estrogen could exert cardioprotective effects by sympatholytic effects reflected in variables of heart rate variability [51] and baroreflex sensitivity [52]. 17β-estradiol may reduce infarct size by attenuating catecholamine-induced injury. Brunvand et al [53] showed that the

adrenoceptor blockade protects against infarct size during reperfusion after prolonged ischemia. Myocardial infarction can alter the control of sympathetic tone which has been linked to an increased extent of infarction and sudden cardiac death after myocardial infarction [54]. Leakage of catecholamines from their storage depots exposes the ischemic myocytes to high concentrations of catecholamine [55]. It appears that modification of autonomic tone of estrogen may have an impact on electrical stability and thereby on infarct size. Besides, because estrogen increases the production of nitric oxide, the resulting increases in nitric oxide concentration may reduce infarct size [15].

4. 4. Clinical implications

Estrogen has been used clinically and has few side effects [56]. Because of the relatively few side effects of estrogen and it is protective even when started after the onset of ischemia, it may be an agent that could be administrated very early to patients who presented with acute myocardial infarction. However, the reperfusion model used differs from the clinical setting. Sudden relief of occlusion by snare instead of a more gradual reperfusion in clinical situations may have influenced the ensuing amount of free radicals. Thus, the clinical benefits of such a reduction in infarct size should be tested in future clinical trials.

4. 5. Study limitations.

There are several limitations to our study. First although the sample size was small, it had the power to detect differences of infarct size as a primary end point. Second, long-term heart function was not an outcome measure, and we do not know whether the limitation of infarct size would be accompanied by an improved mortality. Treatment with free radical scavengers only delay free radical-induced cell death and the initial salvaged tissue undergoes necrosis later in the reperfusion phase ("delayed reperfusion injury"). Such a mechanism could occur if free radical production continued after drug level decreased below the therapeutic levels. Another limitation is technical problems influencing the accuracy of coronary blood velocity [57,58]. Error in Doppler velocity measurements are related to the shape of the velocity profile, the angle of incidence and position of the sample volume and should be minimal in our animals with patent coronary arteries. Paired measurements of coronary blood flow velocity obtained with an intracoronary catheter and an epicardial Doppler probe have correlated highly [57]. It is unlikely that significant error was introduced into the velocity measurements.Fourth, a limitation of measurements of free radicals based on blood measurements is that the changes observed may not correlate with those in various tissue compartments [59]. For reactive species such as plasma hydrogen peroxide, there may even be differences between different points in the circulation [60]. Fortunately, lipid peroxides and conjugated dienes are relatively stable within the circulation, so that peripheral venous levels differ little at any given time-point [61]. Thus in the present study measurements of TBA-reactive material and diene conjugates in femoral artery samples were taken to represent circulating levels in general.

七. Conclusion.

This is the first study which provides evidence that late administration of estrogen prior to coronary reperfusion reduced infarct size after myocardial ischemia as effectively as estrogen administrated when given before coronary ischemia. The beneficial effects of estrogen in physiological concentrations appear to be mediated, at least in part, through the antioxidant activity of estrogen.

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Table 1. Changes of hemodynamics, coronary blood flow and estradiol at different fixed times among Controls, Early and Late Groups

	Control (n = 6)	*Early (n = 6)*	*Late (n = 6)*
Baseline			
HR (bpm)	192 ± 30	193 ± 25	201 ± 20
MBP (mm Hg)	124 ± 23	131 ± 20	125 ± 15
RPP (bpm x mm Hg)	308 ± 98	323 ± 37	307 ± 30
LVEDP (mm Hg)	9 ± 4	9 ± 3	7 ± 4
Coronary blood flow (ml/min)	39 ± 5	42 ± 5	43 ± 4
Estradiol (pg/ml)	56 ± 12	47 ± 6	58 ± 8
Occ, 30 min			
HR (bpm)	189 ± 30	201 ± 29	207 ± 16
MBP (mm Hg)	120 ± 26	127 ± 20	106 ± 19
RPP (bpm x mm Hg)	302 ± 107	325 ± 45	311 ± 51
LVEDP (mm Hg)	11 ± 4	15 ± 7	18 ± 8
Coronary blood flow (ml/min)	3 ± 2	2 ± 3	2 ± 3
Reperfusion, 30 min			
HR (bpm)	188 ± 22	188 ± 27	203 ± 12
MBP (mm Hg)	118 ± 23	122 ± 25	118 ± 26
RPP (bpm x mm Hg)	296 ± 95	299 ± 64	301 ± 50
LVEDP (mm Hg)	15 ± 3	17 ± 7	15 ± 7
Coronary blood flow (ml/min)	52 ± 7	62 ± 9	59 ± 11
Reperfusion, 60 min			
HR (bpm)	190 ± 18	177 ± 23	187 ± 11
MBP (mm Hg)	123 ± 15	128 ± 13	120 ± 25
RPP (bpm x mm Hg)	293 ± 59	270 ± 43	252 ± 88
LVEDP (mm Hg)	15 ± 6	18 ± 10	14 ± 6
Coronary blood flow (ml/min)	55 ± 6	63 ± 8	62 ± 12
Reperfusion, 120 min			
HR (bpm)	184 ± 19	179 ± 29	187 ± 13
MBP (mm Hg)	127 ± 24	126 ± 13	124 ± 14
RPP (bpm x mm Hg)	294 ± 85	275 ± 42	292 ± 33
LVEDP (mm Hg)	16 ± 5	14 ± 8	16 ± 5
Coronary blood flow (ml/min)	57 ± 9	63 ± 11	62 ± 8
Estradiol (pg/ml)	56 ± 11	526 ± 67	641 ± 84

Data are expressed as mean ± SD. HR: heart rate; LVEDP: left ventricular end-diastolic pressure; MBP: mean blood pressure; SBP: systolic blood pressure.

Early group: 17β-estradiol was given intravenously 15 min before coronary occlusion Late group: 3 min before coronary reperfusion following 60- min ischemia