EXPERIMENTAL STUDIES

Hypoxic Injury of Immature Cardiac Myocytes Under Various Hypothermic Conditions Using an In Vitro Cell-Culture Model

Hiroyuki Orita, M.D., Manabu Fukasawa, M.D., Hideaki Uchino, M.D.
Tetsuro Uchida, M.D., Satoshi Shiono, M.D.
and Masahiko Washio, M.D.

The purpose of this study was to evaluate the functional and biochemical effects in immature cardiac myocytes under hypoxic and hypothermic conditions. Cardiac myocytes were isolated from neonatal rat ventricles and cultured for 4 days, after which $12.5 \times 10^5$ myocytes/flask were incubated under 3% hypoxic conditions at 4°C, 10°C, 15°C, 20°C, 25°C, and 37°C for 6, 12, and 24 h. After each hypoxic incubation, creatine kinase (CK) and lactate dehydrogenase (LDH) were measured in the incubation medium. The myocytes were then cultured for an additional 24 h at 37°C to evaluate the recovery of the myocyte beating rate. In the 4°C and 37°C groups, the myocyte beating rate recovery markedly decreased with increasing incubation times from 78.1% and 97.2% at 6 h to 0.0% and 38.4% of the control, which was the beating rate prior to hypoxic incubation, at 24 h, respectively. However, in the 10°C, 15°C, and 25°C groups, this value decreased significantly only at 24 h. In the 20°C group, beating rate completely recovered in 24 h. A marked increase was found in the release of CK and LDH in the 4°C group from 28.5 mIU/flask and 232.9 mIU/flask at 6 h to 83.7 mIU/flask and 640.7 mIU/flask at 24 h, respectively. However, in the 25°C and 37°C groups, this release was significantly increased only at 24 h. In the 15°C and 20°C groups, no significant increases were observed over 24 h. Below 15°C, hypothermia induced cellular damage both functionally and biochemically, and the greatest damage was observed at 4°C. Above 25°C, the damage was due to hypoxia. Thus, a temperature of 15°C to 20°C appears to be suitable for hypothermic preservation of immature myocardium.


In cardiac surgery, cardiac preservation during the neonatal period remains controversial since immature myocardium shows different responses to various environmental stimuli, including hypoxia and hypothermia, than adult myocardium. In addition, immature myocardium has been shown to be inherently more resistant to ischemic injury than adult myocardium.1–3

Previously, we reported that hypothermia at 4°C induced immature myocyte injury which was diminished or accelerated by various cryopreservation solutions, and demonstrated that an in vitro cell-culture system could provide a useful model for evaluating cellular injury under various preservation conditions.4–6 The purpose of the present study was to evaluate the functional and biochemical effects in immature cardiac

Key words:
Immature cardiac myocyte
Hypoxic injury
Hypothermic injury

(Received September, 29 1994; accepted January 12, 1995)
The Second Department of Surgery, Yamagata University School of Medicine,
This work was supported by a grant from the Japanese National Education Ministry.
Mailing address: Hiroyuki Orita, M.D., The 2nd Department of Surgery, Yamagata University School of Medicine, Iida-nishi, Yamagata City, 990-23, Japan

Japanese Circulation Journal Vol. 59, June 1995 347
myocytes under hypoxic and hypothermic conditions. The functional effects in myocytes were evaluated in terms of myocyte beating rate recovery and the biochemical effects were evaluated in terms of CK and LDH release into incubation medium, which has been shown to be indicative of myocyte injury under various preservation conditions$^4$–$^8$.

MATERIALS AND METHODS

Isolation of Cardiac Myocytes

Cardiac myocyte cultures were prepared as previously described$^4$–$^6$,$^9$ Briefly, hearts were removed from 1- to 2-day-old neonatal Wistar male rats. The ventricles from 20 hearts were minced into fine fragments, which were then placed in a 50-ml flask containing 10 ml of 0.1% collagenase (Wako Chemical, Tokyo) in 0.025 mol/L Hepes-buffered minimum salt solution (MSS, Gibco, Grand Island, NY). The flask was agitated for 60 min at 37 ℃. The resuspended cells and small aggregates were passed through a wire-mesh screen to remove large aggregates and debris. The cellular filtrate was suspended in 0.025 mol/L Hepes-buffered MCDB 107 medium (Kyokuto Pharmaceutical, Tokyo) containing 5% fetal calf serum (FCS) (Flow Labs, Rockville, MD) and placed into 75-cm² tissue culture flasks which were incubated for 60 min at 37 ℃. Under these conditions, differential cellular adherence was used to separate cardiac myocytes from fibroblasts. After incubation, unattached cells were resuspended in MCDB 107 containing 2% FCS, transferrin (10 μg/ml, Sigma, St Louis, MO), and insulin (10 μg/ml, Sigma) (>95% myocytes). Myocytes were identified by their beating activity, which began on the 1st day of culture.

Hypoxic Incubation of Cardiac Myocytes under Various Hypothermic Conditions

Cardiac myocytes were cultured in 25-cm² flasks at 37 ℃, and the medium was changed daily for 4 days. A myocyte concentration of 2.5 × 10⁶ cells/ml was chosen and the total number of cells/flask was 12.5 × 10⁵ cells$^4$–$^6$,$^9$ On the 4th day of culture, the myocytes were incubated in MCDB medium at 37, 25, 20, 15, 10 and 4 ℃ for 6, 12 and 24 h, using a N₂-CO₂ incubator constructed from a hypothermic incubator (LNL-111, ESPEC CO LTD, Osaka), and the oxygen concentration was adjusted to 3%. In our apparatus, oxygen concentrations were easily maintained as low as 3%, even under hypothermic conditions at 4 ℃. However, it was very difficult to keep oxygen concentrations below 2%, since large amounts of N₂-CO₂ gas were needed. Thus, we used an oxygen concentration of 3%. After each hypoxic incubation, the medium was changed and the myocytes were incubated at 37 ℃ for an additional 24 h in a humidified atmosphere of 5% CO₂ : 95% air. Triplicate flasks were evaluated for each group and the experiment was repeated an additional six times. All animals received humane care in compliance with the “Principles of Laboratory Animal Care” formulated by the National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86–23, revised 1985).

Cardiac Myocyte Beating Rate Recovery After Hypothermic Incubation

Cardiac myocytes isolated from neonatal rat ventricles formed confluent monolayers by the fourth day of culture, at which time they almost all beat synchronously and steadily at a constant frequency, which was maintained for 5 days of culture$^4$,$^5$,$^9$ Therefore, the myocyte contraction rate for each flask was observed and the mean contraction or beating rate of 10 myocytes/culture flask was determined microscopically. The myocyte beating rate was observed immediately before and 24 h after hypoxic incubation. The recovery ratio of the myocyte beating rate was expressed as a percentage of the control, which was as the beating rate before hypoxic incubation.

CK and LDH Release From Cardiac Myocytes After Hypothermic Incubation

At each time point in hypoxic incubation, creatine kinase (CK) and lactate dehydrogenase (LDH) release from cultured cells were measured spectrophotometrically using CK or LDH assay kits (Unikirate CK or LDH, Chugai Inc., Tokyo) and a spectro-
photometer (RaBA 3030, Chugai Inc, Tokyo). CK and LDH were expressed in international units (mIU) per culture flask.

Statistical Analysis
Data were initially evaluated by a rankit analysis to determine the distribution, then by a Kruskal-Wallis one-way analysis of variance to examine the differences within the groups, and finally by the Mann-Whitney U test. Non-parametric tests were used because some data did not show normal distributions. Results are presented as the mean ± the standard deviation (SD) and differences were considered significant if the p value was less than 0.05.

RESULTS

Beating Rate Recovery After Hypoxic Incubation Under Various Hypothermic Conditions
The myocyte beating rate just prior to hypoxic incubation was 253.6 ± 16.2 beats /min with a range of 241.9 ± 21.4 to 264.2 ± 27.9 for all groups. After 3% hypoxic incubation under various hypothermic conditions, the cultures were allowed to recover for 24 h at 37°C, and the myocyte beating rate was then measured and compared to the rate prior to hypoxic incubation (percent beating rate recovery). In the 4°C group, myocyte beating rate recovery markedly decreased with increasing incubation times from 78.1 ± 16.8 % at 6 h to 12.4 ± 14.5 % at 12 h (p < 0.001). No beating was detected at 24 h. In the 10°C and 15°C groups, the 24-h incubations showed significantly lower recoveries (53.4 ± 16.9%, p < 0.005, and 78.9 ± 14.7%, p < 0.025, respectively) than the corresponding 12-h incubations (81.5 ± 16.5% and 93.7 ± 9.5%, respectively). In the 20°C group, myocyte beating rate recovery was complete even after 24 h of hypoxic incubation (88.6 ± 16.1%), and there were no significant differences among the three incubation times. However, in the 25°C group, myocyte beating rate recovery in the 24-h incubation was significantly less than that in the 12-h incubation (66.1 ± 18.3% vs 87.3 ± 17.2%; p < 0.025). Moreover, in the 37°C group, the beating rate recovery significantly decreased with increasing incubation time to 59.1 ± 18.6% at 12 h (p < 0.001), to

Japanese Circulation Journal Vol.59, June 1995
38.4±4.9% at 24 h (p<0.01) (Fig. 1).

On the other hand, in the 6-h incubation group, only the beating rate recovery in the 4°C incubation (78.1±16.8%; p<0.025) was significantly decreased compared to the 10°C incubation. In the 12-h incubation group, the beating rate recoveries in the 4°C and 37°C incubations (12.4±14.5%, p<0.001, and 59.1±18.6%, p<0.01) were significantly less than those in the 10°C and 25°C incubations, respectively. Furthermore, in the 24-h incubation group, no detectable beating was observed at 4°C. Beating rate recovery increased significantly with increasing incubation temperature, with a peak value at 20°C, and then significantly decreased again to 38.4±4.9% at 37°C (p<0.01 vs 25°C) (Fig. 1).

**LDH Release From Cardiac Myocytes After Hypoxic Incubation Under Various Hypothermic Conditions**

In the 4°C group, the release of LDH from cardiac myocytes measured immediately after hypoxic incubation showed a marked increase over 24 h from 232.9±71.7 mIU/flask at 6 h to 640.7±94.9 mIU/flask at 24 h (p<0.001 vs 12 h). In the 10°C group, the 24-h incubation showed significantly higher LDH levels (305.8±74.7 mIU/flask) (p<0.025), than the 12-h incubation (208.7±65.8 mIU/flask). However, in the 15°C and 20°C groups, no significant increases were observed during 24 h of hypoxic incubation (from 197.7±92.0 mIU/flask to 261.8±75.6 mIU/flask, and 203.1±44.5 mIU/flask to 284.3±97.3 mIU/flask, respectively). In the 25°C and 37°C groups, the 24-h incubations showed significantly greater LDH levels (340.7±83.4 mIU/flask, p<0.05, and 480.1±45.9 mIU/flask, p<0.001, respectively) than the corresponding 12-h incubations (248.7±73.1 mIU/flask and 263.3±103.9 mIU/flask, respectively) (Fig. 3).
On the other hand, in between the 6-and 12-h incubation groups, no significant differences were observed among the various incubation temperatures, although the 4°C incubations at 6 and 12 h showed higher LDH levels (232.9 ± 71.7 mIU/flask and 298.3 ± 98.5 mIU/flask, respectively) than incubations at other temperatures. The greatest LDH levels in the 24-h incubation group were also observed at 4°C (640.7 ± 94.9 mIU/flask) (p < 0.001 vs 10°C), which markedly decreased to 261.8 ± 2.1 mIU/flask at 15°C, and then increased gradually with increasing incubation temperature to 480.1 ± 45.9 mIU/flask at 37°C (p < 0.001 vs 25°C) (Fig. 3).

**DISCUSSION**

Immature myocardium has been shown to be more resistant to hypoxia or ischemia than adult myocardium, and hypothermia is known to reduce the harmful effects of hypoxia or ischemia on the immature myocardium.\(^1 – 3,12\) However, severe hypothermia at 4°C induces cellular damage during the neonatal period.\(^4,5\) In this study, we evaluated the functional and biochemical change in immature myocytes after 3% hypoxic preservation at various temperatures ranging from 4°C to 37°C in vitro.

The 20°C group showed complete protection from hypoxic and hypothermic injury for 24 h. However, a decline in myocyte functional activity, in terms of the percent recovery of the beating rate 24 h after hypoxic incubation, become apparent at 24 h in the 15°C and 25°C groups, while in the 4°C and 37°C groups beating rate recovery decreased significantly with increasing incubation times to 0 and 38%, respectively. In addition, in the 24-h incubation group, the beating rate recovery increased with increasing incubation temperature, reached a peak value at 20°C, and then decreased. On the other hand, in the 6-h incubation group, only the 4°C incubation showed a significantly less recovery than the other temperatures. Thus, these results suggest that below 15°C, hypothermia might induce functional cellular damage with the greatest damage being observed at 4°C, while above 25°C, damage might be due to hypoxia. Therefore, hypothermic preservation at 20°C may give the best functional protection to immature myocytes from hypoxic or hypothermic injury over 24 h. Although observations of beating rate may not reflect myocyte function as accurately as would contractility measurements, evaluation of the recovery ratio may be a useful index for monitoring myocyte function because myocytes respond rapidly to various environmental and chemical stimuli.\(^6,13,14\)

Measurement of biochemical markers, CK and LDH, confirmed the functional observations in that decreased beating rate recovery correlated with an increased release of both CK and LDH. The 15°C and 20°C groups showed complete protection from hypoxic and hypothermic injury over 24 h. However, in the 4°C, 10°C, 25°C, and 37°C groups, the levels of both enzymes significantly increased at 24 h, with the highest levels in the 4°C group. Moreover, in the 24-h incubation group, CK and LDH release decreased with increasing incubation temperature, with a minimum value at 15°C, and then gradually increased. Thus, hypothermia at a temperature of 15°C to 20°C may provide the best protection for immature myocytes from hypoxic or hypothermic injury both functionally and biochemically. In addition, the changes in CK and LDH levels associated with incubation temperatures and times were milder than those in the beating rate recovery. The beating rate recovery was assessed 24 h after hypoxic and hypothermic incubation, while the levels of both enzymes were measured just after incubation. Therefore, reoxygenation after hypoxic incubation appears to accelerate hypoxic or hypothermic injury.

The precise mechanisms of hypothermic injury in immature myocardium are not yet well understood. However, several investigators have postulated that hypothermia may increase free cytosolic calcium ion (derived from the sarcoplasmic reticulum and influxing through the sarcolemma), and depress its efflux via the sodium-calcium exchange mechanism which in turn induces cardiac muscle contracture and increased cellular damage.\(^15 – 17\) Furthermore, hypothermia itself has been shown to induce direct damage of the sarcolemma.\(^4,5\) The precise mechanisms by which neonatal myocardium tolerates hypoxic or ischemic conditions are not well known. However, previously
proposed protective mechanisms of immature myocardium include (1) higher glycogen stores and glycolytic capacity that provide fuel for greater anaerobic ATP production, (2) decreased degradation or efflux (or both) of ATP, (3) lower baseline contractile and energy requirements, (4) a possible difference in sarcolemmal resistance to hypoxia and subsequent calcium influx, (5) increased activity of amino acid metabolism, such as that of glutamate and aspartate and (6) greater resistance to acidosis because of increased intracellular buffering capacity. In this study, hypoxia showed less detrimental effects on immature myocytes than hypothermia below 10°C. Thus, severe hypothermia appears to have a greater effect on immature myocytes than ischemia or hypoxia, although hypothermia at a temperature of 15°C to 20°C reduced the harmful effects of hypoxia.

The model presented here differs from in vivo immature myocardium consisting of myocytes and non-myocytes such as fibroblasts and endothelial cells in that in vivo myocytes are not directly exposed to hypoxic and/or hypothermic conditions. Although our results may not directly apply to in vivo myocardial preservation, this procedure provides a well-defined myocyte-culture system that is a useful model for evaluating the direct effects, such as cytotoxicity, of various environmental or chemical stimuli, including hypothermia and hypoxia. In addition, the 3% hypoxia tested here may not be a sufficient to induce hypoxic cellular injury. Therefore, further experiments under even more hypoxic conditions will be necessary to clearly elucidate the effects of hypoxia on immature myocytes under various hypothermic conditions.

In conclusion, below 15°C, hypothermia induced cellular damage both functionally and biochemically, with the greatest damage observed at 4°C. Above 25°C, damage was caused by hypoxia. Thus, a temperature of 15°C to 20°C appears to be suitable for hypothermic preservation of immature myocardium.

Acknowledgments

The authors wish to thank Mr. Joseph D. Campeau for his assistance in preparing this manuscript.

REFERENCES


