Journal of the American Heart Association

ORIGINAL RESEARCH

Synergistic Effects of Moderate Therapeutic Hypothermia and Levosimendan on Cardiac Function and Survival After Asphyxia-Induced Cardiac Arrest in Rats

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BACKGROUND: This study investigated whether levosimendan, an inotropic calcium sensitizer, when combined with moderate therapeutic hypothermia, may exert synergistic benefits on post–cardiac arrest myocardial dysfunction and improve outcomes.

METHODS AND RESULTS: After 9.5-minute asphyxia-induced cardiac arrest and resuscitation, 48 rats were randomized equally into 4 groups following return of spontaneous circulation (ROSC), including normothermia, hypothermia, normothermia–levosimendan, and hypothermia–levosimendan groups. For the normothermia group, the target temperature was 37°C while for the hypothermia group, the target temperature was 32°C, both of which were to be maintained for 4 hours after ROSC. Levosimendan was administered after ROSC with a loading dose of 10 μg/kg and then infused at 0.1 μg/kg per min for 4 hours. In the hypothermia–levosimendan group, left ventricular systolic function and cardiac output increased significantly, whereas the heart rate and systemic vascular resistance decreased significantly compared with the normothermia group. Also, the concentrations of interleukin 1β at 4 hours post-ROSC and the production of NO between 1 hour and 4 hours post-ROSC were reduced significantly in the hypothermia–levosimendan group compared with the normothermia group. The 72-hour post-ROSC survival and neurological recovery were also significantly better in the hypothermia–levosimendan group compared with the normothermia group (survival, 100% versus 50%, χ^2 test, P=0.006).

CONCLUSIONS: Compared with normothermia, only combined moderate therapeutic hypothermia and levosimendan treatment could consistently improve post–cardiac arrest myocardial dysfunction and decrease the release of pro-inflammatory molecules, thereby improving survival and neurological outcomes. These findings suggest synergistic benefits between moderate therapeutic hypothermia and levosimendan.

Key Words: brain injury ■ cardiac arrest ■ cardiac dysfunction ■ hypothermia ■ inflammation ■ levosimendan ■ survival

Globally, out-of-hospital cardiac arrest (CA) strikes an estimated 44 people per 100 000 annually.[1] The prognosis following out-of-hospital CA remains dismal, with only 2.2% to 10.7% of patients[1] able to survive to hospital discharge.

Lemiale et al[2] indicated that 68% of resuscitated patients experienced post-CA shock, accounting for 35% of the mortality rate, with most occurring during the initial 3 days following cardiopulmonary resuscitation (CPR). Post-CA myocardial dysfunction can lead to hemodynamic instability and poor recovery of neurological function.[3] Post-CA myocardial dysfunction was reversible so that accelerating its recovery may improve survival and neurological outcomes.[4]

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Supplementary Materials for this article are available at https://www.ahajournals.org/doi/suppl/10.1161/JAHA.120.016139

For Sources of Funding and Disclosures, see pages 10 and 11

JAHA is available at: www.ahajournals.org/journal/jaha

CLINICAL PERSPECTIVE

What Is New?

- Combined moderate therapeutic hypothermia and levosimendan treatments could synergistically improve post–cardiac arrest myocardial dysfunction, and consequently survival and neurological outcomes, compared with respective treatment.
- Most hemodynamically adverse effects of respective moderate therapeutic hypothermia or levosimendan treatment were cancelled out in the combined treatments with only hemodynamically beneficial effects left.
- The proinflammatory process following cardiac arrest and cardiopulmonary resuscitation, including elevated interleukin-1β concentration and increased NO production, may be mitigated by the combined treatments.

What Are the Clinical Implications?

- Combined moderate therapeutic hypothermia and levosimendan treatments may be applicable to more patients, because most adverse effects of either treatment alone were not observed with combined treatment.
- For patients experiencing post–cardiac arrest hemodynamic instability, combined moderate therapeutic hypothermia and levosimendan treatments may prevent hypothermia-induced hypotension and thus benefit those patients neurologically.

Nonstandard Abbreviations and Acronyms

CA	cardiac arrest
CO	cardiac output
CPR	cardiopulmonary resuscitation
CVP	central venous pressure
dP/dt_{40}	dP/dt at a left ventricular pressure of 40 mm Hg
HT-Levo	hypothermia with levosimendan
K_{ATP} channel	adenosine triphosphate-sensitive potassium channel
LV	left ventricle
MAP	mean arterial pressure
MTH	moderate therapeutic hypothermia
MW	Mann–Whitney
NT-Levo	normothermia with levosimendan
ROSC	return of spontaneous circulation
SVR	systemic vascular resistance

Cellular energy depletion during CA leads to failure of the membrane Na/K ATPase pump and subsequent intracellular sodium overload,[5] which in turn causes massive intracellular calcium influx through the Na/Ca exchanger. This cytosolic calcium overload impairs myocardial contractility by decreasing the calcium sensitivity of contractile proteins.[6] As a nonadrenergic inotropic calcium sensitizer,[7] levosimendan exerts its inotropic effect principally via binding to the calcium-saturated troponin C of the myocardial thin filament,[7] and may improve post-CA myocardial dysfunction.[8] Levosimendan also produces vasodilatory effects mediated by ATP-sensitive potassium (K_{ATP}) channels,[8] further decreasing the afterload. Despite these favorable hemodynamic effects, few studies have investigated the use of levosimendan for post-CA syndrome.[9]

Guidelines[10] recommend a target temperature of between 32°C and 36°C for treating post-CA syndrome despite the optimal temperature being unknown.[11,12] Moderate therapeutic hypothermia (MTH; 32–33.9°C) reduces the metabolic rate and induces bradycardia, thereby protecting the post-CA ischemic myocardium.[13] However, MTH has complex and opposing effects on the myocardium and myocardial contractility. Therefore, hypothermia was often considered a potential cause of hypotension, leading to the exclusion of patients with hemodynamic instability from the implementation of MTH.[11,12]

In the current study, we investigated whether combined MTH and levosimendan treatments have synergistic effects on improving post-CA myocardial dysfunction and outcomes.

MATERIALS AND METHODS

This animal study was approved by the Institutional Animal Care and Use Committee (approval number 20150352) and conducted according to the Guide for the *Care and Use of Laboratory Animals* by the US National Institutes of Health. All rats were housed in a rodent facility with a 12 hour-light/12 hour-dark cycle and had ad libitum access to food and water before the experiment. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Design and Setting

A randomized animal study was designed to investigate the synergistic effects of MTH and levosimendan treatments in a CA/CPR rat model (Figure 1). Rats were randomized into the following 4 groups by drawing lots: normothermia (NT), hypothermia (HT), normothermia with levosimendan (NT–Levo), or hypothermia with levosimendan (HT–Levo). Targeted temperature management was initiated after the return

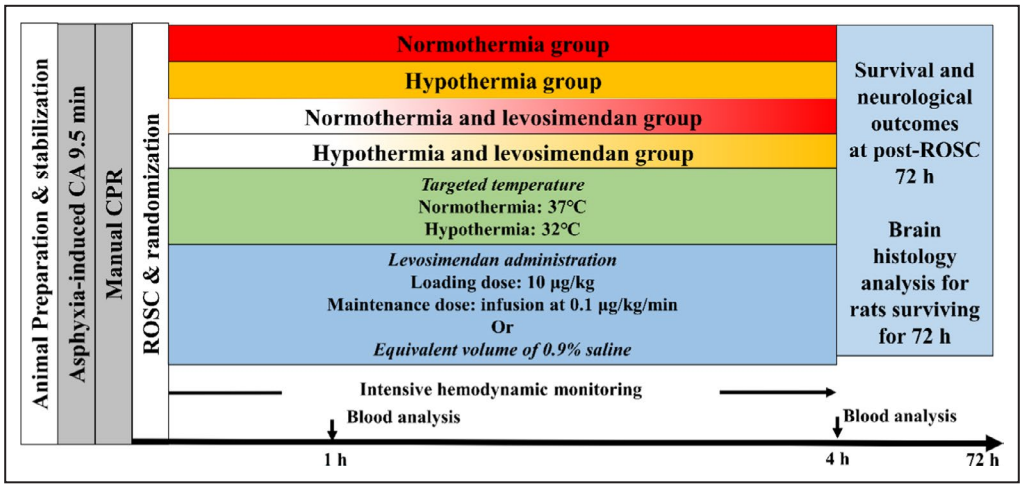

Figure 1. The study design and protocol for inducing cardiac arrest, resuscitation, treatments, and monitoring.
CA indicates cardiac arrest; CPR, cardiopulmonary resuscitation; and ROSC, return of spontaneous circulation.

of spontaneous circulation (ROSC) and maintained for 4 hours: for the HT group, the target temperature was 32°C while for the NT group, the target temperature was 37°C. The needed cooling for MTH was provided by external means. In brief, small electrical fans were activated, and ice packs were applied to bilateral sides of the head and body immediately after ROSC.[14] The target temperature of 32°C was usually achieved within 30 minutes.[15] During the rewarming process, the temperature was kept at the rate of 0.5°C/h when intubated and rewarmed naturally at room temperature after extubation. The temperature of rats receiving MTH usually achieved 37°C at the 10th through 12th hour after initiation of rewarming.[15] Levosimendan (Sigma–Aldrich, St. Louis, MO) was administered intravenously after ROSC with a loading dose of 10 μg/kg and then infused at 0.1 μg/kg per min for 4 hours. For groups not receiving levosimendan, an equivalent volume of 0.9% saline was administered.

Animal Preparation

The preparation of the asphyxia-induced CA/CPR rat model has been detailed previously.[14,16] In brief, 13-week-old male Wistar rats were anesthetized with intraperitoneal pentobarbital (30 mg/kg).[14,16] Anesthetic monitoring, including testing rear foot reflexes, was performed before any surgical procedure; if required, additional pentobarbital (10 mg/mg) would be administered intravenously every 30 minutes.[17] After tracheal intubation, mechanical ventilation (Flexivent EC-VF-2; Scireq Scientific Respiratory Equipment Inc, Montreal, Canada) was initiated with a tidal volume of 0.8 mL/100 g body weight, a frequency of 90 breaths/min, and an inspired oxygen fraction of 100%. Arterial blood pressure and central venous pressure (CVP) were measured through the right femoral artery and right jugular vein, respectively. Left ventricle (LV) pressure was measured with a saline-filled PE-50 tube inserted through the right carotid artery and advanced to the LV.[14,16] Fluids and medications were administrated through the right jugular vein. A PC-based data acquisition system (PowerLab; ADInstruments, Colorado Springs, CO) was used to record physiologic data. A thermocatheter for measuring blood temperature was placed into the left femoral artery and advanced to the thoracic aorta by a fixed-length method. Rats were excluded before the induction of CA if they had a mean arterial pressure (MAP) < 80 mm Hg, experienced surgical bleeding or underwent surgical instrumentation for longer than 40 minutes.

Asphyxia-Induced CA

After stabilization of rats, the ventilator was turned off and the endotracheal tube was clamped to induce CA, which was defined as MAP ≤10 mm Hg. CPR was started after asphyxia for 9.5 minute with 1 dose of intravenous epinephrine (0.01 mg/100 g) followed by manual chest compressions (200 beats/min). During CPR, the ventilator was switched on with pre-CA settings. ROSC was defined as a return of supraventricular rhythm with a MAP ≥40 mm Hg for 10 minutes. Rats were excluded if ROSC did not occur within 6 minutes of CPR. After ROSC, the FiO_2 was adjusted to 21%.

Hemodynamic Monitoring

To evaluate post-CA cardiac dysfunction and recovery, hemodynamic parameters were measured 1, 2, 3,

and 4 hours post-ROSC, which included dP/dt at an LV pressure of 40 mm Hg (dP/dt_{40}), LV maximal negative dP/dt, LV systolic pressure, LV end-diastolic pressure, cardiac output (CO), heart rate, MAP, systemic vascular resistance (SVR), and CVP.

Blood Sample Analysis

All available plasma samples collected 4 hours post-ROSC were used to measure interleukin (IL)-1β and IL-6 concentrations with an enzyme-linked immunosorbent assay kit (R&D Systems, Inc., Minneapolis, MN) by following the manufacturer's instructions. To assess the production of NO, plasma samples available at 1 hour and 4 hours post-ROSC were used to measure nitrite/nitrate concentrations with a nitrite/nitrate immunoassay kit (R&D Systems) using Griess reagents according to the manufacturer's instructions.

Survival and Neurological Outcomes

After intensive monitoring for 4 hours, catheters were removed, wounds were surgically closed, and rats were extubated. Rats were intraperitoneally injected with 1 mL pf 0.9% saline 1 hour after extubation and returned to cages with close monitoring for 72 hours. Death was confirmed by the loss of a heartbeat and spontaneous respiratory movement for 2 minutes. Neurological function was assessed by neurological functioning scores for rats (Table S1).[18] Rats were euthanized with a lethal dose of pentobarbital sodium (250 mg/kg) administered intraperitoneally after completing the evaluation of survival and neurological outcomes 72 hours post-ROSC.

Brain Histological Studies

After euthanasia, the harvested left hemispheres of rats surviving for 72 hours were immediately fixed in 4% formaldehyde and 0.1 mol/L phosphate buffer. Hematoxylin and eosin staining was used to examine brain histological injuries. In each sample, 1 field was randomly selected in the respective cornu ammonis regions of the hippocampus, including CA1, CA2, and CA3, for quantitative comparison by 400× magnification, and expressed as a percentage of damaged/total neurons.

Statistical Analysis

Based on a comparison between NT and HT–Levo groups, at least 11 rats in each group were necessary to demonstrate a difference in 72-hour survival (NT group: 35%; HT–Levo group: 90%) with a power of 80% at the 5% level (MedCalc, version 19.0.7; MedCalc Software, Ostend, Belgium). Therefore, 12 rats were randomly assigned to each group.

Categorical data were expressed as counts and proportions, and continuous data were expressed as mean±SD. Categorical data were compared using a χ^2 test. Continuous data were compared using a Mann–Whitney (MW) test or Kruskal–Wallis test with post hoc Dunn's test, depending on the number of compared groups. Time-based measurements were compared with generalized estimating equations, with the NT group used as the reference. The differences between 1 hour and 4 hours post-ROSC total nitrite concentrations, calculated as 4-hours concentration minus the 1-hour concentration of total nitrite, were compared using simple linear regression, with the NT group used as the reference. Kaplan–Meier plots were used to demonstrate survival curves. Logistic regression analysis was conducted to investigate the interaction between MTH and levosimendan treatment on the primary outcome of 72-hour survival. A 2-tailed *P* value <0.05 was considered significant. All tests were conducted with GraphPad Prism Version 8.2.1 (GraphPad Software, La Jolla, CA) except that generalized estimating equation, simple linear regression, and logistic regression analysis were conducted using SAS 9.4 software.

RESULTS

Baseline Characteristics of Resuscitated Rats

After achieving ROSC, a total of 48 rats were equally randomized into NT, HT, NT–Levo, and HT–Levo groups (Figure 2). Body weight, and duration of CA and CPR were not significantly different between the 4 groups (Table S2).

Hemodynamic Parameters

Comprehensive comparisons of hemodynamic parameters are provided in Table and several representative parameters are provided in Figure 3. Compared with the NT group, LV systolic function, represented by LV dP/dt_{40}, recovered better in the HT–Levo group (*P*=0.002). In contrast, LV diastolic function, represented by LV maximal negative dP/dt, was not significantly different between NT and HT–Levo groups (*P*=0.114). In comparison with the NT group, CO improved significantly in the HT–Levo group (*P*<0.001) despite the heart rate being significantly slower in the HT–Levo group (*P*=0.002). The SVR was significantly reduced in the HT–Levo group compared with the NT group (*P*<0.001), whereas MAP did not significantly differ between these 2 groups (*P*=0.199). Significant differences were not apparent between NT and HT–Levo groups with regard to LV systolic pressure, LV end-diastolic pressure, and CVP.

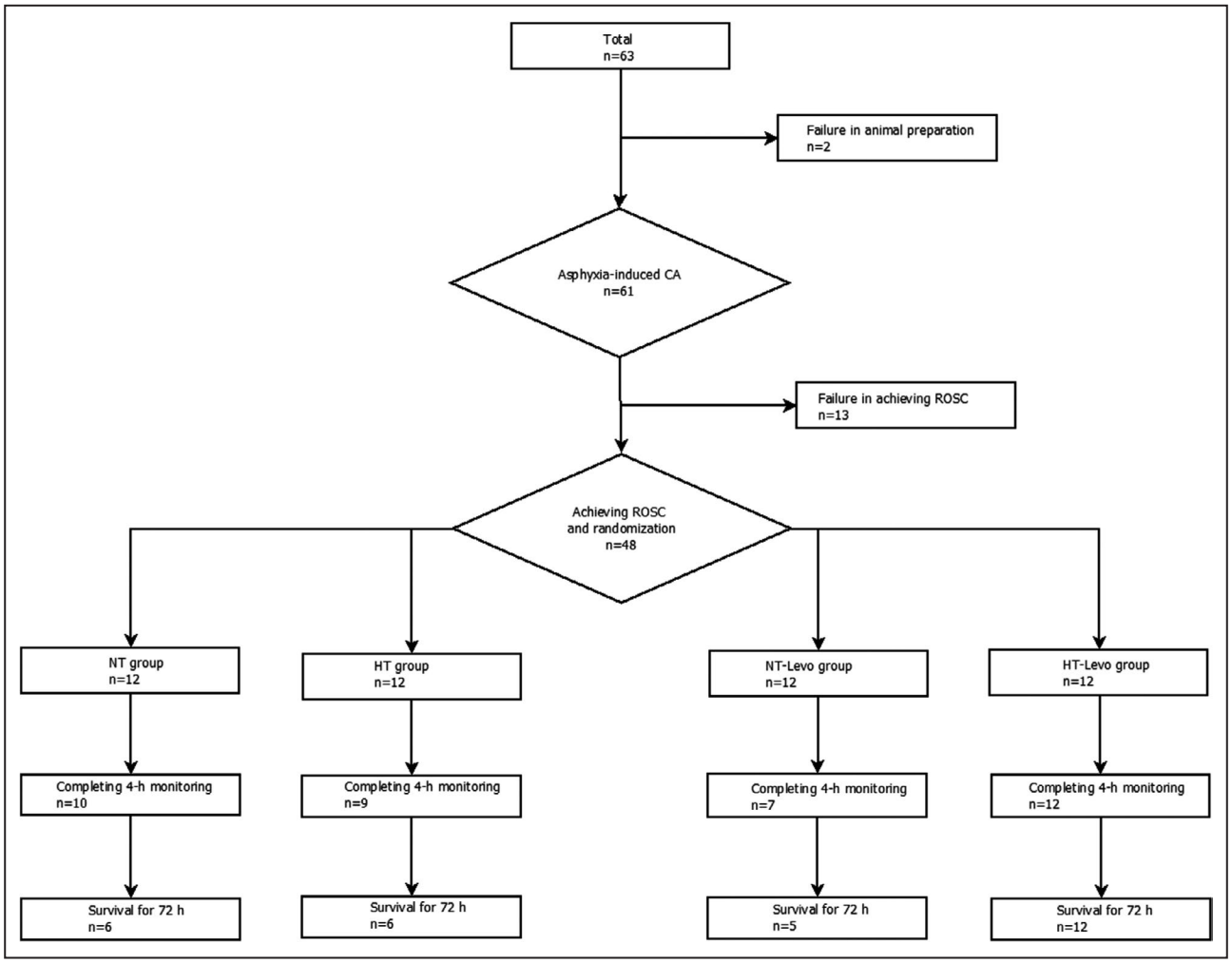

Figure 2. Flow diagram of experimental group randomization.
CA indicates cardiac arrest; HT, hypothermia; Levo, levosimendan; NT, normothermia; and ROSC, return of spontaneous circulation.

Blood Sample Analysis

As shown in Table S3, significant differences in the concentrations of 4-hour IL-1β were noted among the 4 groups (Kruskal–Wallis test, P=0.048), whereas differences were not significant in concentrations of 4-hour IL-6 (Kruskal–Wallis test, P=0.845). In a post hoc analysis, the concentration of 4-hour IL-1β was significantly lower in the HT–Levo group compared with the NT group (67.4±15.6 pg/mL versus 113.3±31.1 pg/mL, Dunn's test, P=0.026). Moreover, in comparison with the NT group, the difference between 1 h and 4 h post-ROSC total nitrite concentrations was significantly reduced in the HT–Levo group (Table S4, simple linear regression test, P=0.046).

Survival Outcome and Neurological Function Assessment

Survival status was observed for up to 72 hours (Figure 2: flow diagram; Figure 4: Kaplan–Meier plot). The 72-hour survival was significantly higher in the HT–Levo group than in the NT group (100% versus 50%, χ^2 test, P=0.006), whereas 72-hour survival of the HT group (50% versus 50%, χ^2 test, P>0.999) or NT–Levo group (42% versus 50%, χ^2 test, P=0.688) was not significantly different from that of the NT group. The logistic regression analysis indicated significant interaction between MTH and levosimendan treatment on 72-hour survival (P=0.045). As shown in Figure 5, the mean 72-hour neurological functioning score of the HT–Levo group was significantly higher than that of the NT group (8.3±5.1 versus 4.3±5.4, MW test, P=0.036), whereas the mean 72-hour neurological functioning scores of the HT group (5.5±5.8 versus 4.3±5.4, MW test, P=0.446) or NT–Levo group (3.5±5.2 versus 4.3±5.4, MW test, P=0.714) were not significantly different from that of the NT group.

Histological Studies for Brain Damage

Following hematoxylin and eosin staining, post-CA neuronal damage manifested predominantly

Table. Hemodynamic Data and Cardiac Function Before Cardiac Arrest and After Cardiopulmonary Resuscitation

Time Point	Pre-Arrest	ROSC	1 h	2 h	3 h	4 h	GEE *P* Value
Left ventricular dP/dt_{40}, mm Hg/s							
NT	9107.4±1614.9	4372.2±949.9	5190.2±1238.1	5096.9±837	5212.5±960.8	4732.2±924.4	Ref
HT	9153.9±1408.9	3982.9±946.7	5063.3±1783.8	5630.4±1759.1	5588.4±1374.2	5405±1484.2	0.705
NT-Levo	9507.4±943.4	4656.1±1430.4	5461.9±1583.1	5588.2±885.6	5739.7±1288	6378.3±1260.2	0.046*
HT-Levo	9252.3±1255.7	4325.9±1018.6	6592.9±1403.8	6762.4±1765.3	6555.8±2121	6728±2159.3	0.002*
Left ventricular maximal negative dP/dt, mm Hg/s							
NT	−10471.3±2539.2	−4599.4±1201.8	−4836.4±1484.7	−4663.1±954.8	−4987.7±947.7	−4502.8±994.3	Ref
HT	−9931±2156.3	−4090.7±1138.5	−4621.2±1904	−5125.6±1897.2	−5226.7±1511.5	−4787±1326.5	0.821
NT-Levo	−10616.1±1874.2	−4751.5±1577.3	−4527±1216.6	−4847.9±1101.1	−5321.4±1456.9	−5836.6±1390.9	0.296
HT-Levo	−10477.5±1777.1	−4511.2±1334.9	−5819.2±1846.6	−5996.6±2278.2	−5526.6±2272.1	−5582±2189.8	0.114
Cardiac output, mL/min							
NT	152.3±17.6	NA	61.7±11.9	61.5±12.4	61.3±11.8	61.7±8.7	Ref
HT	146.6±29	NA	79±22.8	73.9±22.7	79±22.5	72.7±22.1	0.016*
NT-Levo	155.7±25.1	NA	72.8±28.9	63.8±16.5	70.6±24	81.9±29.6	0.013*
HT-Levo	152.3±26	NA	101.7±19.4	85.7±18.9	83.2±19.6	88.3±19.2	<0.001*
Heart rate, beat/min							
NT	399.8±30.4	311.3±35.9	339.1±28.6	346.8±28	358.4±35.7	359.6±37.6	Ref
HT	413.7±44.6	280.3±43	288.2±51.8	298.6±39.2	297.5±41.8	302.9±40.1	<0.001*
NT–Levo	418.9±28	312.3±36.5	336.8±41.6	360.9±37.5	375±35.4	393.4±29.2	0.251
HT-Levo	404.4±35.4	283.5±30.3	308.8±41.8	314±43.4	312.4±44.5	310.5±45.5	0.002*
Mean arterial pressure, mm Hg							
NT	128.8±14.1	69.7±15.6	72±17.2	73.8±11	72.5±16.8	69.6±12.5	Ref
HT	127.9±15.9	69.5±12.8	75.3±23.7	81.2±22.8	85.3±16.1	85.7±16.3	0.098
NT-Levo	137.9±9.5	75.2±18.6	67.6±18.6	70.1±15.3	75.3±20	78.1±11.4	0.441
HT-Levo	129.5±14.1	70.8±16	85.1±19.9	79±20.4	73.6±23.5	76.7±19.9	0.199
Systemic vascular resistance, mm Hg·min·mL^{-1}							
NT	0.9±0.2	NA	1.3±0.6	1.2±0.3	1.3±0.3	1.2±0.2	Ref
HT	0.9±0.1	NA	1.2±0.2	1.2±0.3	1.2±0.3	1.3±0.5	0.851
NT-Levo	0.9±0.2	NA	1±0.3	1.2±0.4	1.1±0.3	1.1±0.4	0.064
HT-Levo	0.9±0.2	NA	0.9±0.2	1±0.2	1±0.2	0.9±0.2	<0.001*
Left ventricular systolic pressure, mm Hg							
NT	158.1±18.1	97.7±14.4	97.4±15.6	99.9±13.6	101.5±8.7	98.5±10.0	Ref
HT	154.8±18.6	96.9±11.7	100.0±21.4	103.7±18.9	111.5±12.7	111.5±10.9	0.259
NT-Levo	163.1±12.1	101.1±15.4	95.1±14.8	97.0±13.6	101.6±17.8	105.9±11.7	0.824

(*Continued*)

Table. Continued

Time Point	Pre-Arrest	ROSC	1 h	2 h	3 h	4 h	GEE *P* Value
HT-Levo	158.5±15.0	99.8±13.8	108.2±16.3	106.1±17.9	102.2±22.2	101.2±16.5	0.400
Left ventricular end-diastolic pressure, mm Hg							
NT	2.0±1.6	7.9±7.1	4.3±3.7	6.6±6.0	8.5±7.0	8.8±4.8	Ref
HT	1.7±2.5	9.0±9.7	4.5±3.8	5.5±4.2	7.0±5.1	7.2±5.4	0.721
NT-Levo	1.8±1.6	7.5±4.6	4.5±3.4	4.6±3.8	4.4±4.0	4.0±4.2	0.147
HT-Levo	1.6±1.5	9.0±5.9	4.1±2.9	3.1±2.3	2.8±2.5	2.8±2.3	0.066
Central venous pressure, mm Hg							
NT	−0.4±1.9	2.3±2	1.4±1.7	1.6±2.1	1.2±3	1.5±2.6	Ref
HT	0.3±1.2	1.8±1.7	1.7±1.6	2.2±1.5	2.1±2.6	2.5±1.6	0.513
NT-Levo	0.1±1.6	2.2±1.8	1.5±2.1	1.4±2.4	1.5±2.2	0.8±3	0.819
HT-Levo	0.7±1.5	2.4±1.2	2.2±1.8	2.2±1.8	2±1.9	1.7±2	0.409

dP/dt_{40} indicates dP/dt at a left ventricular pressure of 40 mm Hg; GEE, generalized estimating equations; HT, hypothermia; Levo, levosimendan; NA, not available; NT, normothermia; and ROSC, return of spontaneous circulation.
*Asterisks indicate statistical significance.

as nuclear condensation and vacuolar alterations around the nucleus, termed eosinophilic-like changes (Figure 6). However, in rats surviving for 72 hours, the proportions of neurons demonstrating these changes in the hippocampus were not significantly different between HT–Levo and NT groups (31.2%±6.9% versus 42.2%±11.4%, MW test, P=0.200).

DISCUSSION

Main Findings

This study demonstrated that combined MTH and levosimendan treatments could improve post-CA myocardial dysfunction, and consequently survival and neurological outcomes. Furthermore, the lower IL-1β concentration and decreased NO production in the HT–Levo group suggested that the proinflammatory process following CA and CPR may be mitigated by the combined treatment, also contributing to improved outcomes.

Improving Post-CA Myocardial Dysfunction and Survival

Asphyxia is one of the major causes of CA in adults,[19] especially among patients with nonshockable rhythms.[12] Compared with ventricular fibrillation–induced CA, asphyxia-induced CA leads to more severe post-CA myocardial dysfunction and a shorter survival time.[20] As a cardiac myofilament calcium sensitizer,[7] levosimendan increases myocardial contractility without worsening post-CA intracellular calcium overload,[21,22] which was hypothesized to complement MTH in treating post-CA myocardial dysfunction.

As shown in Table, compared with the NT group, LV dP/dt_{40} increased significantly in both NT–Levo and HT–Levo groups. Therefore, the augmented LV systolic function observed in the HT–Levo group may have been predominantly caused by the administered levosimendan. Furthermore, compared with the NT group, the HR decreased significantly in both HT and HT–Levo groups. In previous studies, levosimendan was shown to increase the HR.[23] Therefore, the bradycardia noted in the HT–Levo group was mainly caused by the MTH. Taken together, the summed effects of levosimendan-augmented LV systolic function and MTH-induced bradycardia may lead to more pronounced increases in CO in the HT–Levo group compared with increases caused by MTH or levosimendan alone.

As for SVR, the opposing effects of MTH-induced vasoconstriction[13] and levosimendan-induced vasodilation[24] converged toward vasodilatation, as observed in the HT–Levo group showing significantly decreased SVR compared with the NT group. This vasodilatory effect may be beneficial for post-CA myocardial

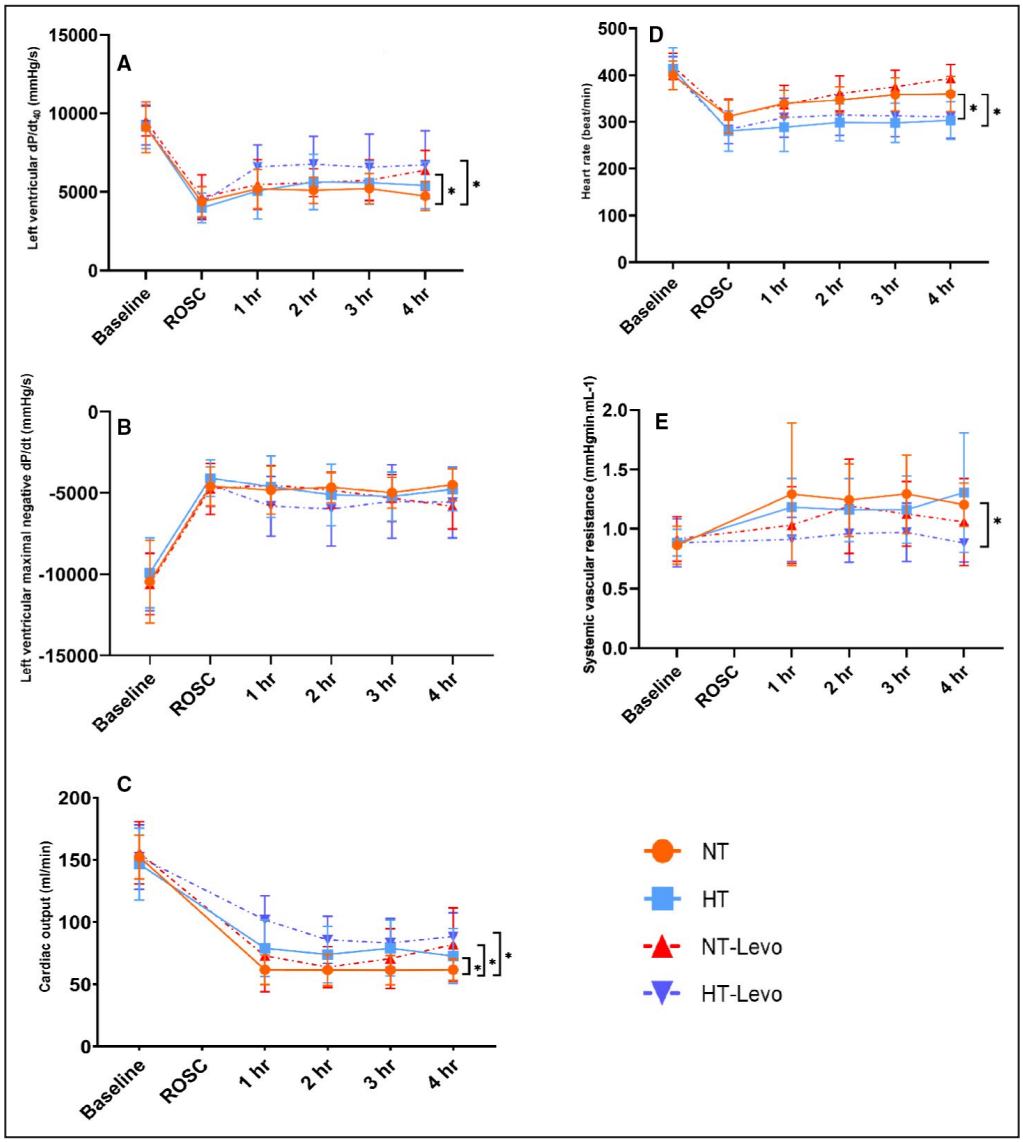

Figure 3. Hemodynamic parameters of experimental groups from baseline to 4 h after cardiac arrest and cardiopulmonary resuscitation.
dP/dt_{40} indicates dP/dt at a left ventricular pressure of 40 mm Hg; HT, hypothermia; Levo, levosimendan; NT, normothermia; and ROSC, return of spontaneous circulation. *Asterisks indicate statistical significance.

dysfunction by decreasing afterload, and contribute to improved organ perfusion along with an augmented CO. Interestingly, in a study by Kelm et al,[23] levosimendan-induced vasodilatation caused a decrease in MAP, which was nonetheless not observed in our HT–Levo group. This unaltered MAP suggested that the augmented CO may offset the decrease in SVR in the HT–Levo group. Moreover, LV end-diastolic pressure and CVP in the HT–Levo group were not significantly different from those of the NT group. MTH was expected to increase venous return because of peripheral vessel constriction,[25] which could have increased LV end-diastolic pressure and CVP. Again, the unchanged values in LV end-diastolic pressure and CVP may be caused by the opposing effects of levosimendan-induced vasodilatation in the venous side.[26]

Taken together, such beneficial effects observed in the HT–Levo group highlighted the synergistic effects of combined MTH and levosimendan treatments on post-CA myocardial dysfunction and hemodynamics.

Reduced Post-CA Brain Injuries

Post-CA brain injury features impaired cerebral microcirculation and compromised cerebral blood flow.[27] Therefore, the accelerated recovery from post-CA myocardial dysfunction observed in the HT–Levo group may contribute to a better neurological

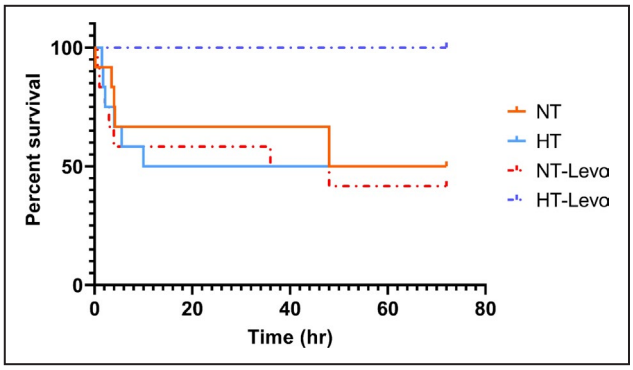

Figure 4. Kaplan–Meier survival curves of randomized groups.
HT indicates hypothermia; Levo, levosimendan; and NT, normothermia.

outcome. Moreover, levosimendan may activate K_{ATP} channels present in both cerebral large arteries and microvessels to induce cerebral vasodilatation.[28] Kelm et al[18] noted post-CA cerebral hypoperfusion could be recovered by levosimendan in an asphyxia-induced CA rat model. Also, compared with normothermia, Gong et al[29] indicated that MTH also preserved cerebral cortex microcirculation and reduced the cerebral oxygen extraction ratio after CPR in a rat model. Therefore, MTH and levosimendan may synergistically improve cerebral energy homeostasis, leading to a better neurological outcome in the HT–Levo group. In a brain histological analysis, because we could only harvest the brain tissue of rats that survived for 72 hours, the numbers of available samples for comparisons were limited, resulting in underpowered comparisons. Nonetheless, a trend towards neurons being less damaged in the HT–Levo group was noted (Figure 6).

Decreased Post-CA Inflammatory Response

Decreased inflammatory responses may also contribute to improved hemodynamics, survival, and neurological outcomes. After a period of ischemia–reperfusion, an inflammatory response ensues, with proinflammatory cytokines, such as those of the IL-1 family,[30] released in a large amount.[31] Such inflammatory and immunologic responses occur especially during reperfusion and are accompanied by free radical production. This can result in substantial injuries via phagocytosis by macrophages, the release of toxic products, and the continued activation of immune reactions in a vicious cycle. Numerous animal experiments and several clinical studies have shown that MTH suppresses ischemia-induced inflammatory reactions and the release of proinflammatory cytokines.[32] Levosimendan also has anti-inflammatory effects.[33] In

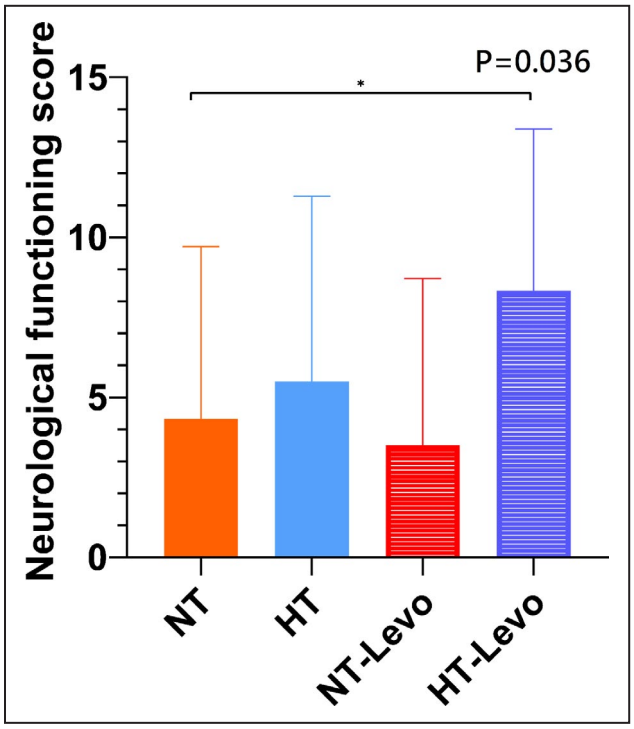

Figure 5. Neurological functioning scores of experimental groups 72 hours after cardiac arrest and cardiopulmonary resuscitation.
Data are presented as the mean±SD; *P*=0.036 by Mann–Whitney test. HT indicates hypothermia; Levo, levosimendan; and NT, normothermia.

a rat model of middle cerebral artery occlusion, levosimendan reduced postreperfusion cerebral edema, an inflammatory response and the expression of tumor necrosis factor-α.[33]

Brain inflammation is mainly caused by the activation of glial cells, which produce various proinflammatory and neurotoxic factors, such as NO. The production of NO was assessed by measuring total nitrite concentrations in our study. Excessive NO production by NO synthase in activated glia may contribute to both neurodegeneration and neuroprotection, depending on the concentration of NO.[34] NO may stimulate soluble guanylyl cyclase, thereby decreasing the concentration of intracellular calcium and terminating chain-propagating lipid radical reactions caused by oxidative stress.[35] In contrast, reactive nitrogen species can also exert cytotoxic effects, which are mainly mediated by the highly reactive oxidant peroxynitrite, a chemical product of NO, and superoxide anions.[36] MTH can decrease NO production.[37] Levosimendan also reduced nitrosative stress markers[38] in patients with heart failure. In summary, both a decreased IL-1β concentration and reduced NO production in the HT–Levo group highlighted the synergistic beneficial effects of combined MTH and levosimendan treatment. Nevertheless, in our study, we could not confirm the

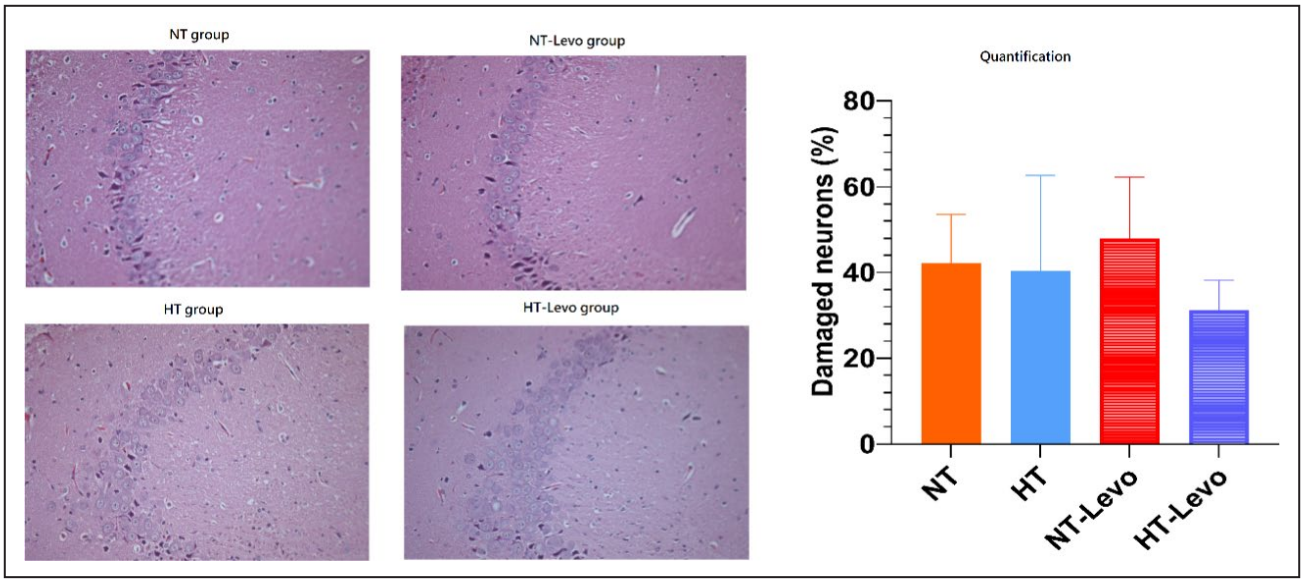

Figure 6. Histological studies of brains in experimental groups.
Hematoxylin and eosin staining of hippocampus from rats surviving 72 hours after cardiac arrest. Microscopic magnification 400×. Data are presented as the mean±SD. HT indicates hypothermia; Levo, levosimendan; and NT, normothermia.

source of NO, ie, whether or not NO was produced from constitutively expressed NO synthases (including neuronal NO synthase and endothelial NO synthase) or inducible NO synthase. Although from the perspective of time sequence, most of the early NO production may come from the neuronal or endothelial NO synthase, inducible NO synthase may still contribute to this increased production through complex cross-talk among these NO synthases.[39] The exact mechanisms of how combined MTH and levosimendan treatment leads to reduced NO production should be further explored.

Study Limitations

First, current guidelines[10] recommend a target temperature of between 32°C and 36°C for post-CA patients. We selected 32°C as the target temperature in the hope of maximizing the potential benefits of MTH. However, whether the treatment effects noted in the HT–Levo group can be generalized to other target temperatures is unknown. Second, the calculation of sample size was based on a comparison between NT and HT–Levo groups, with a focus on investigating the synergistic effects of MTH and levosimendan. The animal numbers in the current study may not be powered enough to detect the effect of a single treatment in the HT or NT–Levo groups. Finally, our study was performed using healthy animals without any known comorbidities. The impact of underlying diseases, such as heart failure, on the responses to levosimendan treatment was not clear. Further studies in disease models are needed to clarify this issue.

CONCLUSIONS

Compared with normothermia, only combined MTH and levosimendan treatment was consistently shown to improve post-CA myocardial dysfunction and decrease the release of pro-inflammatory molecules, including IL-1β and NO, thereby improving survival and neurological outcomes. These findings highlight the synergistic effects of MTH and levosimendan, which could potentially be translated into a clinical application for post-CA myocardial dysfunction.

ARTICLE INFORMATION

Received February 2, 2020; accepted May 1, 2020.

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Acknowledgments

We thank the staff of the 3rd Core Lab, Department of Medical Research, National Taiwan University Hospital for technical support. The authors acknowledged statistical assistance provided by the Center of Statistical Consultation and Research in the Department of Medical Research, National Taiwan University Hospital.

Sources of Funding

Author Chih-Hung Wang received a grant (105-2628-B-002-009-MY3) from the Ministry of Science and Technology. Author Chien-Hua Huang received a grant (105-2314-B-002-125-MY2) from the Ministry of Science and Technology. Ministry of Science and Technology had no involvement in designing the study, collecting, analyzing or interpreting the data, writing the manuscript, or deciding whether to submit the manuscript for publication.

Disclosures

None.

Supplementary Materials

Tables S1–S4

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Supplemental Material

Table S1. Definition of neurological functioning scores.

Level of consciousness	Corneal reflex	Respirations	Righting reflex	Coordination	Movement/ activity	Score
No reaction to pinching of tail	No blinking	Irregular breathing pattern	No turning attempts	No movement	No spontaneous movement	0
Poor response to tail pinch	Sluggish blinking	Decreased breathing frequency, normal pattern	Sluggish turning	Moderate ataxia	Sluggish movement	1
Normal response to tail pinch	Normal blinking	Normal breathing frequency and pattern	Turns over quickly and spontaneously	Normal coordination	Normal movement	2

Table S2. Baseline characteristics of resuscitated rats.

	NT (n=12)	HT (n=12)	NT-Levo (n=12)	HT-Levo (n=12)	Kruskal-Wallis test *p*-value
Bodyweight, g (SD)	468 (39)	454 (34)	444 (29)	446 (29)	0.303
Cardiac arrest time, s (SD)	279 (52)	282 (61)	274 (51)	259 (52)	0.734
CPR time, s (SD)	134 (27)	127 (17)	121 (24)	128 (18)	0.522

CPR: cardiopulmonary resuscitation, HT: hypothermia, Levo: levosimendan, NT: normothermia, SD: standard deviation.

Table S3. Comparisons of pro-inflammatory cytokines.

Group	4-h Interleukin-1β (pg/mL)	Dunn's test adjusted *p*-value	Kruskal-Wallis test *p*-value	4-h Interleukin-6 (pg/mL)	Dunn's test adjusted *p*-value	Kruskal-Wallis test *p*-value
			0.048			0.845
NT	113.3±31.1	Reference		741.5±219.5	Reference	
HT	91.6±25.4	0.768		688.4±316.1	>0.999	
NT-Levo	104.1±21.5	>0.999		1045±734.5	>0.999	
HT-Levo	67.4±15.6	0.026*		738.6±234.1	>0.999	

HT: hypothermia, Levo: levosimendan, NT: normothermia. *Asterisk marks indicate statistical significance

Table S4. Comparisons of total nitrite concentrations.

Group	1-h total nitrite (μmol/L)	4-h total nitrite (μmol/L)	$\triangle_{\text{4h-1h total nitrite}}$ (μmol/L)	Simple linear regression *p*-value
NT	33.6±4.3	45.8±12.1	12.2±11.2	Reference
HT	32.5±4.7	49.2±10.6	16.7±8.7	0.482
NT-Levo	31.9±6.6	53.9±15.3	22.0±15.1	0.137
HT-Levo	31.4±8.8	30.2±6.5	-1.2±6.9	0.046*

$\triangle_{\text{4h-1h total nitrite}}$: calculated as 4-h concentration minus the 1-h concentration of total nitrite, HT: hypothermia, Levo: levosimendan, NT: normothermia. *Asterisk marks indicate statistical significance