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Effects of levosimendan on calcium transient in norepinephrine-cultured neonatal rat ventricular myocytes

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KEYWORDS
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Action potential duration

This study aimed to explore the changes in calcium transient in the development of heart failure and the effects of levosimendan (LeV) on intracellular calcium dynamics. Cultured neonatal rat ventricular myocytes were divided into four groups: normal, norepinephrine (NE) only (10 μmol/L), NE + LeV1 (0.1 μmol/L), and NE + LeV2 (1 μmol/L). The calcium transients of the myocytes loaded with Fluo-3/AM were observed using a laser scanning confocal microscope. Compared with the control group, the calcium wave in the NE group dispersed, propagated slowly, and exhibited dyssynchrony of Ca^{2+} release. Norepinephrine accelerated the beating rate of the cultured myocytes, decreased the systolic peak Ca^{2+}, and increased the time to peak (Ttp) and decay time (Tau) of calcium transient. Levosimendan increased the synchrony of calcium transient, and reduced Ttp and Tau. In contrast, LeV did not affect the beating rate and systolic peak Ca^{2+}. Both NE-only- and LeV-treated groups did not affect resting Ca^{2+} and calcium transient amplitude of the myocytes. The currents from L-type calcium channel currents did not differ among the groups. Both NE and LeV shortened the action potential duration, but the effect of the latter was more serious than that of the former. Western blot results showed that the sarco/endoplasmic reticulum Ca^{2+}-ATPase 2 (SERCA2) expression decreased in the NE group but increased in the LeV groups. The sodium–calcium exchanger 1 (NCX1) expression increased in the NE group but decreased in the LeV groups. Long-term exposure to NE decreased myocardial contractility by reducing the peak Ca^{2+} of calcium transient and by prolonging and disrupting the conduction of calcium waves. Levosimendan elicits a positive inotropic effect by accelerating the velocity of calcium signal propagation and synchronizing calcium release without increasing calcium influx.

Introduction

The molecular basis of myocardial cells is the induction and release process of Ca^{2+}. In cardiac myocytes, an influx of a small number of Ca^{2+} is observed through L-type calcium channel currents (LTCCs) during an action potential (AP), which triggers Ca^{2+} release from the sarcoplasmic reticulum (SR) via ryanodine receptors (RyR2). The released Ca^{2+} then binds to troponin C (cTnC) in myofilaments, thereby initiating systole. Ca^{2+} is then pumped back into the SR via sarco/endoplasmic reticulum Ca^{2+}-ATPase 2 (SERCA2) and extruded through the sodium–calcium exchanger 1 (NCX1) in a forward mode. Thus, relaxation is induced. Calcium mishandling is a key factor in the pathogenesis of heart failure. Reduced systolic peak Ca^{2+}, increased diastolic Ca^{2+} levels, and prolonged diastolic Ca^{2+} decay rate

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slow the velocity of cardiomyocyte relaxation and decrease contractility. Traditional inotropic drugs, such as phosphodiesterase (PDE) inhibitors, increase cyclic adenosine monophosphate concentration and calcium influx from LTCCs, which trigger the release of calcium from the SR. Thus, intracellular calcium concentration is increased. Calcium overload[1] and cardiac arrhythmia are induced. As a consequence, the clinical applications of these drugs are limited. Levosimendan (LeV) is a new inotropic agent that binds to the amino acid N-terminal of cTnC, thereby stabilizing the combination of cTnC and Ca^{2+} in a calcium-dependent manner[2] without directly affecting the concentration of Ca^{2+} in myocardial cells.[3] Levosimendan exhibits inotropic properties without disrupting ventricular relaxation or inducing Ca^{2+} overload.[4] Calcium mishandling leading to heart failure is mainly due to abnormalities in excitation–contraction coupling. This study aimed to explore the changes in calcium transient in the development of heart failure and the effects of LeV on intracellular Ca^{2+} dynamics. Norepinephrine (NE) is the main catecholamine hormone that increases during the progression of heart failure.[5] Thus, we simulated the environment of heart failure by pre-treating neonatal rat ventricular myocytes with NE for 48 h. The myocytes were then treated with LeV for 48 h.

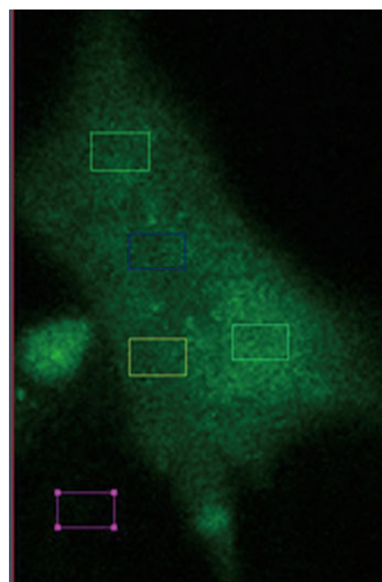

Figure 1 Fluorescence intensity of calcium transient of neonatal rat myocytes stained with Fluo-3/AM was recorded in a spontaneous contraction period. The average fluorescence values of the same size area were collected for analysis.

Methods

Neonatal rat ventricular cardiomyocyte cultures

Neonatal Sprague–Dawley rats were provided by the Experimental Animal Center of Jiangsu Province (Animal Certificate of Conformity: SCXK (Su) 2013-0005). The rats were treated in accordance with ethical standards. Neonatal rat cardiomyocytes were isolated and purified enzymatically under sterile conditions. The hearts of the newborn Sprague–Dawley rats were recovered, and the atrial and macrovascular tissues were removed. The apex of the myocytes were washed thrice in pre-cooled PBS, minced until the pieces were $\sim$0.5 mm^3, and dispersed via digestion with pancreatin (Gibco, 0.1% volume fraction, 5 mL at each time) at 35–37°C by using a magnetic stirrer. The cells in the supernatant were transferred to a tube containing Dulbecco's modified Eagle medium (DMEM), a cell culture medium (Sigma, USA), with 10% foetal bovine serum, streptomycin, and penicillin. This process was repeated seven to nine times until no tissue was left. The tubes were centrifuged at 1000 $\times$ g for 6–8 min at room temperature; the cell pellet was resuspended in 5 mL of the cell culture medium and then incubated in a 5 cm dish for 1 h to inhibit fibroblast contamination. The remaining cells were plated in four 3 cm glass bottom dishes in a humidified 5% CO_2 and 95% O_2 atmosphere. Afterwards, 0.1 mmol/L Brdu was added to inhibit the growth of fibroblasts. The culture medium was replaced every 2–3 days after 24 h. The samples were randomly divided into four groups that were separately treated with NE or LeV.

Detection of calcium transient

The cultured neonatal cardiomyocytes were washed thrice with cold PBS, loaded with 1 mM Fluo-3/AM (Beyotime) for 30 min in an incubator with 5% CO_2, washed with DMEM, and incubated in the complete medium for 30 min. Fluo-3 was excited by using a 488 nm line of argon-ion laser. Fluorescence was detected at a wavelength of 525 nm. A series of frame scan was collected at 80–120 ms/frame and at $\times$63 magnification of oil endoscopy of 10 cells from each group via confocal laser scanning microscopy (Carl Zeiss, Germany) to record calcium transients. The calcium transients were analysed using an AimImageBrowser software in different areas of the same size (*Figure 1*). Calcium transient-related parameters,[6] including calcium transient amplitude, systolic peak Ca^{2+}, resting Ca^{2+}, time to peak (Ttp), decay time (Tau), and beating rate were recorded. The average intensity of fluorescence represented calcium concentration. Data were encoded in Microsoft Excel for further analysis. These data can be converted into motion pictures through AimImageBrowser software.

Action potential duration and LTCC currents recording

Action potential duration (APD) was recorded using an Axopatch200B patch-clamp amplifier (Molecular Devices, Union City, CA, USA) and a Digidata1440A (Molecular Devices, Union City, CA, USA). The dishes (Warner Instrument Corp., Hamden, CT, USA) containing the cultured myocardial cells were washed with an external solution several times to remove macromolecular proteins from the culture media. Single ventricular myocytes with an intact membrane and sharp edges were selected as the recorded cells. Patch electrodes (Sutter Instrument Co., Novato, CA, USA) were fabricated from a borosilicate glass with a micropipette puller (Sutter Instrument Co., Novato, CA, USA). Pipette resistance was set at a range of 2–4 MΩ to minimize voltage errors caused by series resistance. The recorded APD was analysed in a current-clamp mode at a threshold current pulse of 5 ms at the frequency of 1 Hz. Action potential duration at 90% is the duration of AP repolarization at 90% amplitude. The cells were then perfused with the LTCC recording solution. K^+ currents were blocked with Cs^+. The cell was maintained at a holding potential of -80 mV, and then depolarized to a test potential of -40 mV for 200 ms to inhibit T-type Ca^{2+} and Na^+ channel currents. The series resistance was adjusted to 80%. The test potentials ranged from -60 to 70 mV. The experiments were performed at room temperature (20–22°C). Tyrode solution contained 135 mM NaCl, 5.4 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 5 mM HEPES, and

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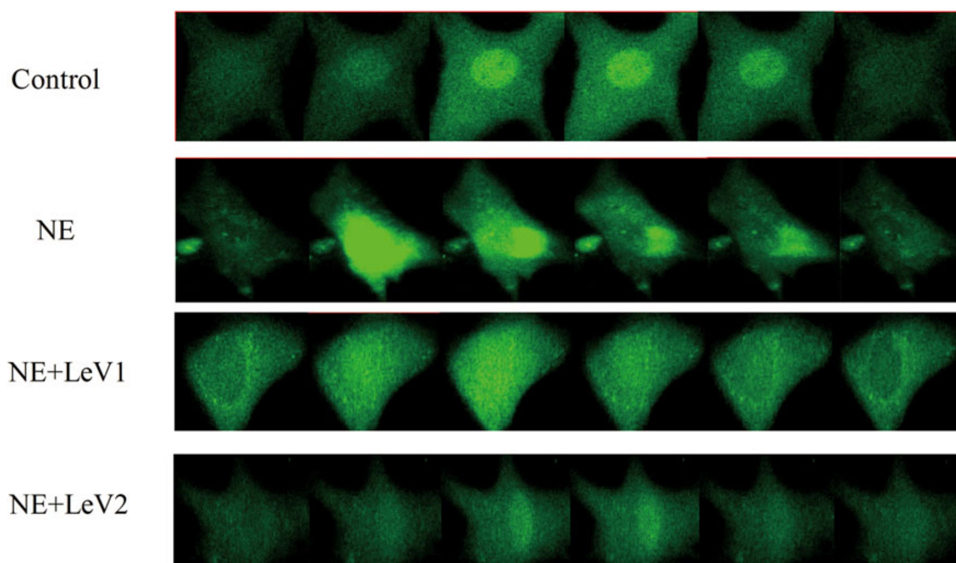

Figure 2 Manifestations of calcium transient. A series of frame scan was collected at ×63 magnification of oil endoscopy. Representative fluorescence images suggested that norepinephrine disrupted the conduction of calcium wave. The images of norepinephrine- and levosimendan-treated groups showed that levosimendan synchronized the calcium release.

5 mM glucose; this solution was maintained at pH 7.4, adjusted with 10 M NaOH. The pipette solution of LTCCs contained 120 mM CsCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM Na_2ATP, 10 mM EGTA, and 10 mM HEPES; this solution was maintained at pH 7.4 by adding CsOH. The bath solution of LTCCs contained 120 mM choline-Cl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 4 mM CsCl, 10 mM HEPES, and 10 mM glucose; pH was adjusted to 7.4 by using 1 M CsOH.

Western blots

The samples were recovered from the dishes and homogenized in a lysis buffer. The homogenate was centrifuged at 15 000 *g* and 4°C for 15 min to remove debris. The supernatant was collected, and the protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). A portion of the protein was added to the sample with 5× buffer and boiled for 10 min. Myocardial samples (20 μg protein per lane) were electrophoretically separated through sodium dodecyl sulfate–polyacrylamide gels (SDS–PAGE) (8% separation gel, 5% stacking gel). The SDS–PAGE-resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes at 30 mA for 2 h in an ice bath. The PVDF membranes containing the transferred proteins were blocked for 2 h with Tris-buffered saline containing 1% Tween-20 (TBST) and 5% skimmed milk. The blocked membranes were then incubated with anti-SERCA2/ATP2A2, anti-NCX1, or anti-glyceraldehyde-3-phosphate dehydrogenase (1:1000) overnight at 4°C and washed thrice with TBST for 5 min. Afterwards, the membranes were washed thrice with TBST, detected using a secondary antibody (rabbit anti-rat IgG-horseradish peroxidase, 1:1000; Jackson, Bioworld), and visualized through an enhanced chemiluminescence assay (Pierce ECL Western Blotting Substrate; Thermo Scientific). Band densities were quantified using the gel analysis programme of ImageJ software (NIH, Bethesda, MD, USA).

Statistical analysis

Data were expressed as mean ± SE. Two-tailed unpaired Student's test and one-way ANOVA were applied when appropriate. Least significant difference was used after rank transformation when variances were inhomogeneous. $P < 0.05$ was considered statistically significant.

Results

Alteration of the calcium transient in the cultured neonatal rat cardiomyocytes

Compared with the control group, the calcium wave in the NE group dispersed, propagated slowly, and exhibited dyssynchrony of Ca^{2+} release (*Figure 2*). Norepinephrine accelerated the beating rate of the cultured cells (26.7 ± 4.3 vs. 11.6 ± 3.6, $P < 0.01$), decreased the systolic peak Ca^{2+} (128.37 ± 65.44 vs. 155.33 ± 61.77, $P < 0.05$), and increased Ttp (0.413 ± 0.324 vs. 0.212 ± 0.050, $P < 0.01$) and Tau (1.162 ± 0.524 vs. 0.722 ± 0.169, $P < 0.01$) of calcium transient. However, no significant differences were observed in the calcium transient amplitude and the resting Ca^{2+}. Norepinephrine in the presence of LeV increased the synchrony of calcium transient and reduced Ttp (0.212 ± 0.044 vs. 0.413 ± 0.324, NE + LeV1 vs. NE, $P < 0.01$; and 0.205 ± 0.062 vs. 0.413 ± 0.324, NE + LeV2 vs. NE, $P < 0.01$, respectively) and Tau (0.735 ± 0.269 vs. 1.162 ± 0.524, NE + LeV1 vs. NE, $P < 0.01$; and 0.753 ± 0.152 vs. 1.162 ± 0.524, NE + LeV2 vs. NE, $P < 0.01$). In contrast, LeV did not affect the beating rate of the cultured cells and systolic peak Ca^{2+}, and did not influence resting Ca^{2+} and calcium transient amplitude. These results demonstrated the mechanism by which LeV increased the rate of myocardial contractility and improved the ventricular contractile function. No concentration differences were observed in the presence of LeV (*Table 1* and *Figure 3*).

Whole-cell patch clamp

LTCC currents play an important role in cardiac excitation–contraction coupling. The current kinetics of whole-cell

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Table 1 Characteristics of the calcium transient ($x \pm s$)

Groups	Peak Ca^{2+}	Resting Ca^{2+}	Amplitude	Ttp	Tau	Beating rate
C ($n = 25$)	155.33 ± 61.77	79.92 ± 38.05	75.41 ± 36.52	0.212 ± 0.050	0.722 ± 0.169	11.6 ± 3.6
NE ($n = 47$)	$128.37 \pm 65.44^{\#}$	67.57 ± 42.59	60.80 ± 39.88	$0.413 \pm 0.324^{\#\#}$	$1.162 \pm 0.524^{\#\#}$	$26.7 \pm 4.3^{\#\#}$
NE + LeV1 ($n = 20$)	132.08 ± 50.34	83.26 ± 46.59	48.82 ± 29.33	$0.212 \pm 0.044^{**}$	$0.735 \pm 0.269^{**}$	25.8 ± 8.9
NE + LeV2 ($n = 47$)	112.44 ± 38.54	55.27 ± 21.36	57.17 ± 33.20	$0.205 \pm 0.062^{**}$	$0.753 \pm 0.152^{**}$	24.3 ± 3.8

$^{\#}P < 0.05$; $^{\#\#}P < 0.01$ (vs. control); $^{**}P < 0.01$ (vs. NE).
NE, norepinephrine; LeV1, 0.1 μmol/L levosimendan; LeV2, 1 μmol/L levosimendan.

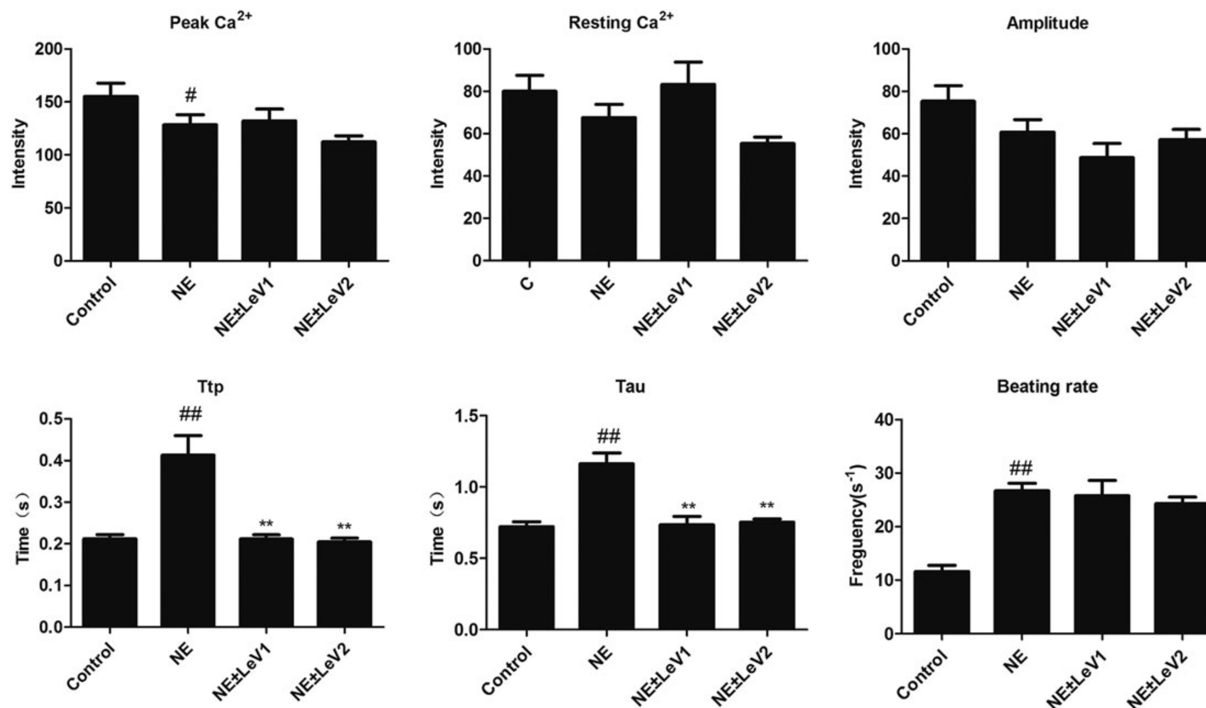

Figure 3 Effects of levosimendan on calcium transient-related parameters and beating rate. The calcium transients were analysed using an AimImageBrowser software in different areas of the same size. Norepinephrine reduced the peak Ca^{2+} of calcium transient and increased the time to peak and decay time, compared with the control group. However, levosimendan decreased the time to peak and decay time, accelerating the velocity of calcium signal propagation. All of them failed to affect amplitude and resting Ca^{2+} of calcium transient. $^{\#}P < 0.05$; $^{\#\#}P < 0.01$ (vs. control); $^{**}P < 0.01$ (vs. norepinephrine).

LTCCs exhibited similar patterns in the control group and NE- and LeV-treated groups (*Figure 4*). This result suggests that LeV did not affect calcium influx. The whole-cell patch clamp revealed that APD_{90} was shorter in the NE groups than in the control group (187.65 ± 11.53 vs. 245.40 ± 18.92, $P < 0.01$). Levosimendan decreased APD_{90} more obviously than NE (7.62 ± 0.72 vs. 187.65 ± 11.53, NE + LeV1 vs. NE, $P < 0.01$; and 8.5 ± 1.11 vs. 187.65 ± 11.53, NE + LeV2 vs. NE, $P < 0.01$). This finding may be related to the myocardial ATP-dependent potassium channels (KATP) opened by LeV (*Table 2* and *Figure 5*).

Myocardial sarco/endoplasmic reticulum Ca^{2+}-ATPase 2 and sodium–calcium exchanger 1 protein expressions

Compared with the myocardial SERCA2 and NCX1 expression levels in the control group, the myocardial SERCA2 expression decreased and the NCX1 expression increased in the NE-treated groups. Levosimendan prevented the decrease in SERCA2 expression and the increase in NCX1 expression after treated with LeV 48 h (*Figure 6*).

Discussion

Amplitude and velocity of calcium transient and myocardial contractility

Calcium transient is the molecular basis of myocardial contraction; the magnitude, velocity, and decay rate of calcium transient directly affect the extent, rate, and diastolic velocity of myocardial contraction.[7] The amplitude and peak Ca^{2+} of calcium transient decreased, the resting Ca^{2+} increased, and the duration was prolonged in most models of heart failure, which induced the reduced myocardial contractility together.[8] The duration was also extended

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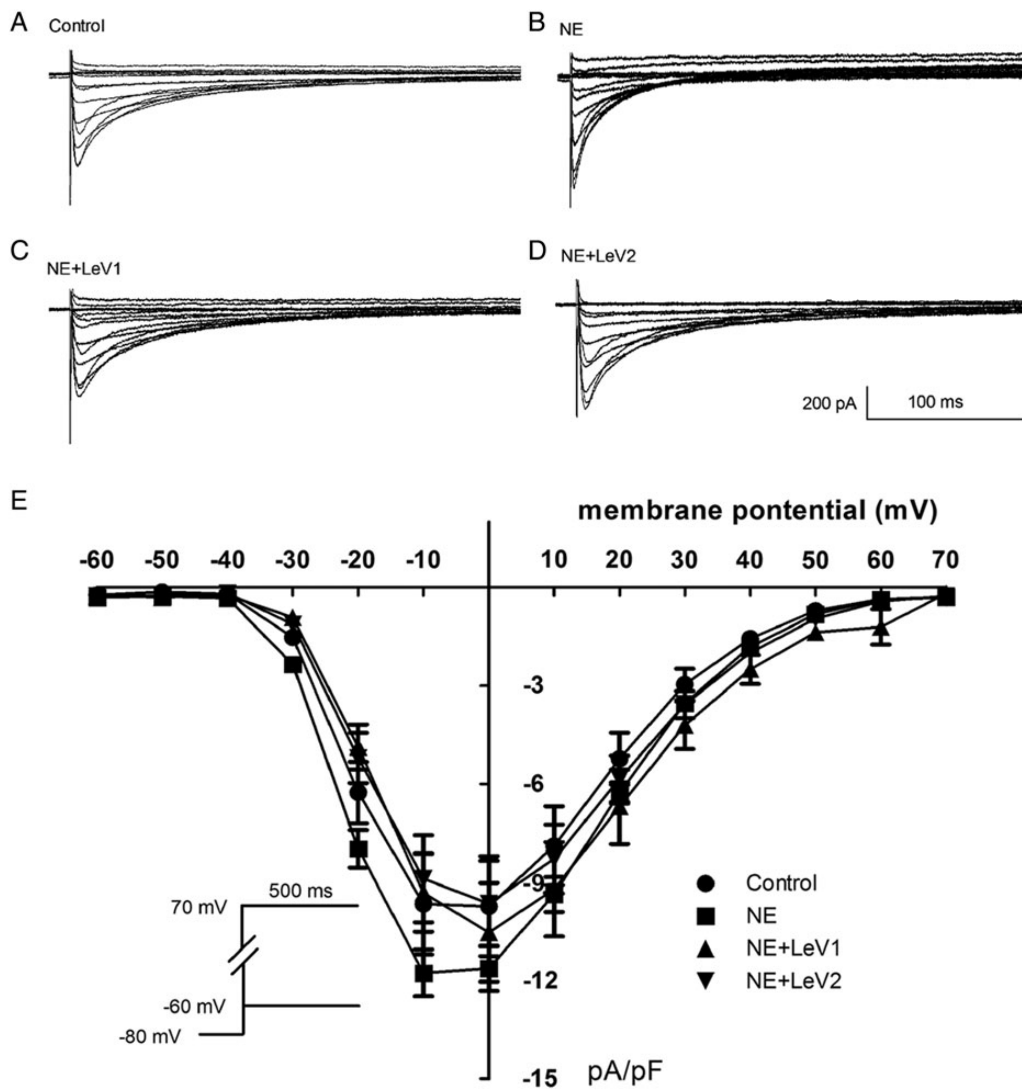

Figure 4 Recording of LTCC currents. (*A–D*) LTCC currents recording in norepinephrine-only- and levosimendan-treated groups. (*E*) Current–voltage relationship for the four groups. No differences were observed among them.

Table 2 Alteration of the action potential duration ($x \pm s$)

	Control	NE	NE + LeV1	NE + LeV2
APD_{90} (ms)	245.4 ± 18.92	187.65 ± 11.53#	7.62 ± 0.72*	8.5 ± 1.11*
n	6	6	5	5

#$P < 0.01$ (vs. control); *$P < 0.01$ (vs. NE).
NE, norepinephrine; LeV1, 0.1 μmol/L levosimendan; LeV2, 1 μmol/L levosimendan.

in our experiment. Time to peak and decay time were increased in NE group. However, the amplitude and resting Ca^{2+} remained unchanged. One hypothesis that can explain our results is that we are unable to classify heart failure. Calcium handling progressively weakens from myocardial hypertrophy to heart failure; thus, the amplitude can be unchanged and diastolic Ca^{2+} does not increase before end-stage heart failure.[9] However, our study failed to fully explain the time at which the intracellular Ca^{2+} dynamics was impaired and arrhythmias occurred. Species, age, and other factors should also be considered.[10] In the present study, we confirmed that the delayed propagation of calcium transient contributed to the decreased contractile force.

Dyssynchronous Ca^{2+} homeostasis and myocardial contractility

We observed that calcium wave propagated in a disorderly manner after NE was administered. The failure of

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excitation–contraction coupling is affected by the amplitude and duration of the calcium transient and the dyssynchrony of Ca^{2+} release.[11] The dyssynchrony of calcium release failed to activate cTnC. Thus, the contraction of the neighbouring sarcomere is likely inhomogeneous, thereby reducing the speed of myocardial contractility and cardiac contractility.[12] Various factors desynchronized Ca^{2+} cycling, including loss and abnormality of T tubules,[13] immaturity of RYR2, variability in Ca^{2+} sparks kinetics,[14] and the AP shape.[15] However, our study did not include related research on these corresponding experimental conditions.

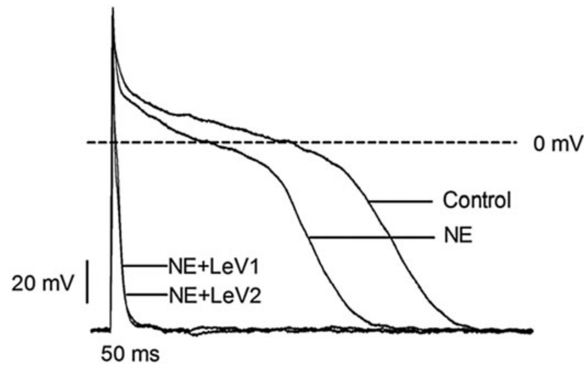

Figure 5 Action potential duration at 90% results in norepinephrine-only- and levosimendan-treated neonatal rat cardiomyocytes. Action potential duration at 90% was shorter in the norepinephrine group than in the control group. Levosimendan shortened action potential duration at 90% more evidently than norepinephrine. The recorded action potential duration was analysed in a current-clamp mode at a threshold current pulse of 5 ms at the frequency of 1 Hz. Action potential duration at 90% is the duration of AP repolarization at 90% amplitude.

Levosimendan and myocardial contractility

Compared with the conduction of calcium wave in the NE-only group, the conduction of calcium wave in the LeV group was more uniform, and Ttp and Tau were shortened after LeV was administered. These results can explain the mechanism by which LeV increases the rate of myocardial contractility and improves heart function. In contrast, LeV failed to influence the systolic peak Ca^{2+}, diastolic Ca^{2+}, and the amplitude of calcium transient. This result indicated that LeV increased the contractility of the cardiac muscle; this contractility may partly be mediated by improving the calcium sensitivity of force contraction without affecting the intracellular calcium ion concentration. Levosimendan is a novel inotropic Ca^{2+} sensitizer, which binds to cardiac cTnC in a calcium-dependent manner.[4] During systole, the intracellular calcium ion concentration increases, and the affinity of cTnC for Ca^{2+} increases. Thus, the actin–myosin cross-bridge conformation rate is accelerated. The muscle contractility rate is also accelerated, thereby improving contractile force. During diastole, the intracellular calcium ion concentration rapidly decreases, and the dissociation rate of actin–myosin cross-bridge increases. Thus, these processes may shorten Tau and positively influence the relaxation rate. Thus, the present study suggested that LeV could improve the systolic function without impairing the ventricular relaxation.

LTCC currents and heart failure

In previous studies, the LTCC currents initially increase and then decrease; afterwards, the LTCC currents remain constant during the development of heart failure.[16,17] We detected the LTCC currents to examine the alteration of

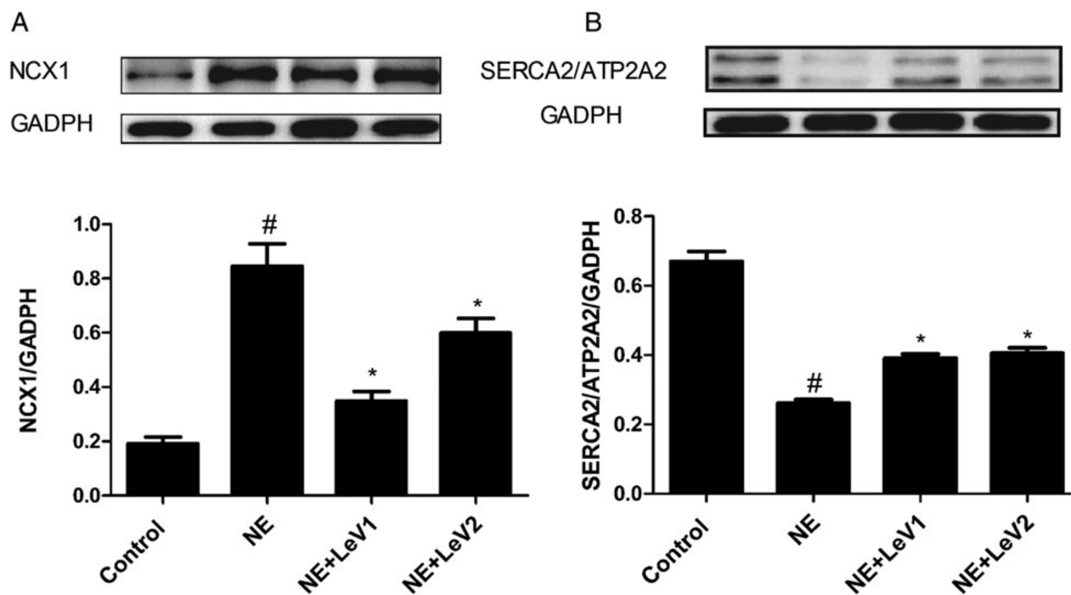

Figure 6 Effect of levosimendan on expression levels of sarco/endoplasmic reticulum Ca^{2+}-ATPase 2 and sodium–calcium exchanger 1. (*A*) Expression of sodium–calcium exchanger 1 increased with norepinephrine-only treatment, compared with the control group. The expression then decreased after treated with levosimendan for 48 h. (*B*) Expression of sarco/endoplasmic reticulum Ca^{2+}-ATPase 2 decreased in norepinephrine-only group, but increased after treated with levosimendan for 48 h. The expression of sarco/endoplasmic reticulum Ca^{2+}-ATPase 2 and sodium–calcium exchanger 1 was normalized to glyceraldehyde-3-phosphate dehydrogenase as the internal control. $^{\#}P < 0.05$ (vs. control); $^{*}P < 0.05$ (vs. norepinephrine).

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intracellular Ca^{2+} concentration. Our study showed that the LTCC currents remained unchanged after these cells were pre-treated with NE. This discrepancy may be due to several parameters, such as disease stage, species type, and experimental conditions. Levosimendan did not also affect the LTCC currents; this finding is consistent with that described in previous studies.

The results of whole-cell patch clamp showed that NE and LeV shortened the APD; the effect of the latter was more evident than that of the former. The APD is possibly prolonged in failing hearts to increase the LTCC currents, particularly at low heart rates. However, the APD is shortened as the heart rate increases.[18] The reduction in APD is more evident in small rodents with relatively rapid heart rates than in large rodents. In our report, the heart rate in NE group was higher than that in control group, and no difference existed between NE- and LeV-treated groups. Thus, the shortened APD may account for no alteration of the LTCC currents in NE- and LeV-treated groups.[18] Levosimendan may open myocardial KATP, thereby shortening the APD more significantly,[19] but the mechanism needs further investigation. LTCC currents can trigger a large-scale Ca^{2+} release from the SR and induce cardiac excitation–contraction. Thus, we deduced that LeV altered the velocity of calcium transient conduction and the formation of calcium wave without increasing the calcium influx. Levosimendan also elicited an anti-arrhythmic effect[3] and promoted myocardial ischaemia and hypoxia by opening the myocardial KATP.[19] An *in vitro* research has also revealed that LeV activates KATP in vascular smooth muscle cells and in the mitochondrial membrane; thus, LeV causes vasodilatory and cardioprotective effects without increasing oxygen demand.[20] However, we have not detected the KATP currents until now.

The SERCA2 expression was reduced and the NCX1 expression was enhanced in NE group, which are consistent with previous studies. Sarco/endoplasmic reticulum Ca^{2+}-TPase 2 and sodium–calcium exchanger 1 are the dominant substances that remove calcium from the cytosol. The decreased SERCA2 protein level and the increased NCX1 expression reduced the SR calcium content; these processes contributed to the dysregulation of intracellular Ca^{2+} cycling and the disruption of ventricular contractile function.[21] Levosimendan promoted these protein levels in failing hearts. This phenomenon is also similar to that observed in other studies on LeV with calcium-handling proteins. However, LeV failed to influence the systolic peak Ca^{2+}, diastolic Ca^{2+}, and the amplitude of Ca^{2+} transient. Other studies believe that ion remodelling occurs earlier than protein abnormalities during the development of heart failure.[22] Changes in the amplitude and velocity of calcium transient could affect the transmembrane ion movement, thereby enhancing the expressions of related proteins in cardiomyocytes. However, whether LeV alters the expression of calcium channel proteins by affecting the ion flow needs to be confirmed.

Levosimendan slightly inhibits PDE enzymes at concentrations of ≥ 1 μmol/L; as a result, LTCCs calcium influx is increased.[23] However, we failed to find the differences between 1 and 0.1 μmol/L LeV. Such differences may be related to species or experimental conditions.

Conclusion

In failing hearts, decreased myocardial contractility may be due to the reduced peak Ca^{2+} of calcium transient and the prolonged and disrupted conduction of calcium waves. Levosimendan elicits a positive inotropic effect by accelerating the velocity of calcium signal propagation and synchronizing calcium release without increasing calcium influx.

Limitations

We failed to examine the expression of other calcium-handling proteins and the currents from NCX1 and KATP. Neonatal rat cardiomyocytes differ from adult rat cardiomyocytes. In data processing, data cannot be perfectly analysed without the use of an appropriate confocal software.

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Conflict of interest: none declared.

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