Functional and Histological Assessment of an Experimental Model of Takotsubo's Cardiomyopathy

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Background—Our objectives were to characterize functional and structural features of an experimental model of Takotsubo cardiomyopathy, and its response to beta-blockers.

Methods and Results—In protocol 1, a dose-finding study: 69 rats received various doses of isoproterenol (ISO) and echocardiographic and histologic parameters were measured on days 2 to 3 or day 8. There were no dose-dependent effects and, out of 69 ISO-treated rats, 40 (58.0%) survived and 29 (42.0%) died within 24 hours. Of survivors, 30 had apical akinesis averaging 12.1±1.6% of the long axis LV circumference. Out of the 40 survivors, 32.5% showed apical akinesis ≥10%, 42.5% showed akinesis<10% and 25% showed no apical akinesis. The basal portion of the LV was always preserved. At 24 hours, histology and ultrastructure showed necrosis, vacuolization, lipid droplets, mononuclear cell infiltration, damaged mitochondria, and edema. On day 8, apical akinesis fully resolved but histologic abnormalities were still present. In protocol 2, rats were randomized to Control; ISO100 mg/kg; propranolol+ISO; and metoprolol+ISO groups. Pretreatment with propranolol and metoprolol improved survival to 90% and 100% respectively, compared with 60% in the ISO group, but did not reduce the incidence and extent of akinesis or the structural damage.

Conclusion—TC can be mimicked in a rat model of ISO exposure that demonstrates apical akinesis on days 2 to 3 with full recovery of systolic regional wall motion abnormality despite the presence of persistent foci of necrosis and fibrosis on day 8. Pretreatment with beta-blockers improved survival but did not affect structural and functional alterations. (*J Am Heart Assoc*. 2014;3:e000921 doi: 10.1161/JAHA.114.000921)

Key Words: electron microscopy • histopathology • isoproterenol • Takotsubo cardiomyopathy

T akotsubo cardiomyopathy (TC) is characterized by acute, transient apical and mid-ventricular akinesia with either a preserved or hypercontractile basal segment, in the absence of epicardial coronary artery obstruction. However, this wall motion abnormality resolves over days to weeks. It is known to be triggered by physical or emotional stress, which elevates the endogenous level of catecholamines. Also, exogenous administration of catecholamines such as epinephrine injec-

tion and disease conditions such as pheochromocytoma induce similar wall motion abnormalities. TC comprises 1 to 2.2% of the suspected cases of acute coronary syndrome. Although the overall prognosis is good, there is a finite acute mortality rate among the TC patients (1 to 1.5%). The current available treatment is primarily symptomatic as the underlying etiology remains elusive. Therefore, to devise rational and targeted therapy there is a need to better understand the pathophysiology of TC following the catecholamine surge.

Recent clinical studies have reported structural alterations in the akinetic region of TC patients using cardiac imaging techniques and subendocardial biopsy analysis. However, experimental models of TC demonstrating the correlation between structural abnormalities and contractile dysfunction at the acute and recovery phase are lacking. Moreover, there is no study that has elucidated the changes in the components of the contractile apparatus at the ultrastructural level. Izumi et al have shown that metoprolol reduces myocytolysis and improves ejection fraction earlier than controls in the i.v. epinephrine induced TC in cynomolgus monkeys. Beta-blockers are also used as a symptomatic treatment during the course of contractile dysfunction in TC patients. However,

there is a lack of substantial evidence that proves their beneficial effect on either structure-function defect or survival at the acute phase. Therefore, the purpose of this study was (1) to better delineate the time course of changes in this model by echocardiography at both the apex and base of the heart; (2) to determine the structural and ultrastructural changes; (3) to determine whether histological abnormalities resolved at the same time as functional abnormalities; and (4) to determine whether pretreatment with beta blockers would prevent the structural and functional abnormalities.

Methods

The study was reviewed and approved by the Institutional Animal Care and Use Committee of Good Samaritan Hospital, and the protocol conformed to the Guide for the Care and Use of Laboratory Animals published by the National Research Council (Eighth Edition, 2011).

Protocol 1

Male Sprague-Dawley rats weighing 250 to 300 g were included in the study. Doses of exogenous isoproterenol were administered to attempt to mimic TC. The doses tested were 25, 50, 85, 100, 170, 200, 400, and 600 mg/kg, administered subcutaneously once or twice at the interval of 24 hours. Repeated dosing of isoproterenol was administered to mimic episodes of stress, which sometimes occur in a repetitive fashion. We attempted to determine whether consecutive increases in catecholamine levels at closer time periods acted synergistically, resulting in further deterioration of regional contractile dysfunction/akinesis associated with Takotsubo cardiomyopathy or an increase in death. Echocardiography was recorded 24 hours after the last dose of ISO (days 2 to 3) to assess the percentage of akinesis of the long axis sub-endocardial circumference of the left ventricle (LV) and fractional shortening at both the apex and base of the heart. The objective of dose titration was to identify the optimum dose, which induces discernible akinesis of the LV in the maximum number of treated animals with a minimal mortality rate. All of the doses induced the same average percentage of akinesis of the LV circumference but twice dosing significantly increased the mortality. Therefore, we selected a single subcutaneous dose of ISO to induce Takotsubo cardiomyopathy in Protocol 2. All the animals underwent surgical procedure for assessment of hemodynamic parameters either 24 hours after the last dose of ISO or 1 week post-ISO (day 8). Hearts were processed separately for triphenyltetrazolium chloride (TTC) staining, hematoxylin & eosin (H&E) staining, Oil red O staining and electron microscopic examination.

Protocol 2

On the basis of Protocol 1, a single dose of ISO 100 mg/kg, s.c. was selected to induce Takotsubo-like akinesis of the apical to mid-ventricular segment.

Since Takotsubo cardiomyopathy has been associated with high plasma catecholamine levels, which act as ligand for the beta-adrenergic receptors, we hypothesized that blocking the action of beta-adrenergic receptors could inhibit akinesis. Therefore, in the second part of the study, rats were randomized into 4 groups: Control (neither ISO nor beta blocker treated) group, ISO-treated group, propranolol pre-treated group (non-selective β-blocker with intrinsic sympathomimetic activity), and metoprolol pre-treated (selective β1 blocker) group. Both propranolol and metoprolol pretreatment groups were injected intraperitoneally with a single dose of propranolol (10 mg/kg) or metoprolol (10 mg/kg) 30 minutes prior to subcutaneous administration of ISO 100 mg/kg. Doses of beta-blocker were based on previous studies assessing the safety and heart rate-reducing properties of the drugs.[1,2] Twenty-four hours after ISO administration, animals underwent surgical procedure for hemodynamic recording and thereafter hearts were excised, stained with 1% 2,3,5-triphenyl tetrazolium chloride (TTC) and stored for hematoxylin and eosin (H&E) and picrosirius red (PSR) staining.

Surgical Protocol

After 24 hours of the last dose of ISO administration or on day 8, surviving animals were anesthetized with intraperitoneal injection of xylazine (5 mg/kg) and ketamine (75 mg/kg). The neck and chest were shaved and cleaned. Animals were ventilated with room air via a rodent respirator. 2D and M-mode echocardiography were recorded to assess the percentage of akinesis and changes in both apical and basal fractional shortening. The right carotid artery was isolated and the catheter was advanced into the left ventricle for recording hemodynamic parameters including mean arterial pressure (MAP), heart rate (HR), left ventricular end-systolic pressure (Pes), left ventricular end-diastolic pressure (Ped), left ventricular peak positive pressure (+dP/dt) and left ventricular peak negative pressure (−dP/dt).

Echocardiography

After 24 hours of the last dose of ISO or day 8, echocardiography was performed using a high-frequency pediatric ultrasound transducer (Philips Sonos 5500 at 12 MHz). This apparatus allows clear imaging of the endocardial surface in both systole and diastole with M-mode and 2D echocardiography. 2D mode was used to record and analyze percentage of apical akinesis of the long axis subendocardial circumference

of the left ventricle. M-mode was used to determine the fractional shortening both at the apex and base of the heart. In addition, we anesthetized the rats with xylazine and ketamine during the echocardiography, which lowers heart rate and allows for better resolution of wall motion.

TTC and Histology

After hemodynamic recording, rats were euthanized with KCl to arrest the heart in the diastolic phase. Hearts were excised and sliced into 4 sections from apex to base. Sections were incubated in TTC for 15 minutes at 37°C for assessment of confluent areas of necrosis. These sections were photographed and stored in 10% formalin for H&E and PSR staining. Semi-quantification of both H&E and PSR stained slides was performed using a scoring system (Tables 1 and 2).

Oil Red O staining

After recording hemodynamic parameters, hearts were excised and washed in cold saline. They were sliced into 3 sections, apical, mid-ventricular, and basal and thereafter, fixed in tissue-freezing medium. Tissue sections were cut into 8-μm slices on a cryostat and mounted on glass slides. After air drying the slides, sections were fixed by incubating in 4% paraformaldehyde for 5 minutes and then washed with phosphate buffered saline. After washing, slides were rinsed with 60% isopropanolol, and then incubated in 0.3% (weight/volume) Oil red O stain prepared in 60% isopropanolol for 15 min, thereafter, rinsed in 60% isopropanolol again. Oil red O stained lipid droplets red in color and nuclei appeared blue with Mayer's hematoxylin stain; thereafter slides were washed with distilled water, mounted, and photographed with a digital camera mounted on a light microscope.

Electron Microscopic Analysis

Three rats that showed significant apical to mid ventricular akinesis on echocardiography 24 hours after ISO 100 mg/kg,

s.c. injection were utilized for perfusion fixation. Animals were anesthetized with xylazine and ketamine i.p. injection. An abdominal incision was made to expose the aorta and inferior vena cava. A catheter was placed in the abdominal aorta positioned towards the heart for perfusion fixation and a nick was made in the inferior vena cava to drain the blood. Phosphate buffer solution was injected for 3 minutes at a pressure equal to the mean blood pressure (122 cm H_2O, 90 mm Hg) to remove blood; thereafter KCl was injected to arrest the heart in a diastolic phase followed by 15 minutes perfusion with modified Karnovsky solution. The fixed heart was excised and ~1 mm × 1 mm × 2 mm-thick slices were cut from the apical region, immersed in modified Karnovsky fixative overnight at 4°C for further fixation and then processed for ultrastructural analysis.

Statistical Analysis

Data were expressed as mean±standard error of mean. Statistical analyses of the echocardiographic and hemodynamic variables were performed by ANOVA followed by post hoc Tukey's test. The proportion of rats surviving in each group was compared using Fisher's exact test. Scoring data was expressed as median [interquartile range] and analyzed by Mann−Whitney test (Non-parametric test). All the statistical analyses were performed by IBM SPSS Statistics 20 (SPSS, Inc.). Statistical significance was considered at the value of $P<0.05$.

Results

Protocol 1

Total number of animals used was 75, out of which 69 rats were treated with ISO and 6 rats were included in the control (no ISO) group (Figure 1). There was no dose–dependent effect of ISO on the percentage of akinesis of the left ventricular circumference (Figure 2). Therefore, all the rats

Table 1. Grading System Used to Score the Histopathological Damage in the ISO-Treated Animals

Score	Myocardial Damage
0	No lesions
0.5	Slight derangement of muscle fibers, few inflammatory cells and vacuoles
1	Focal lesions of the subendocardial portion of the apex and mid-ventricle, inflammatory cells, interstitial edema, vacuolization of myocytes
1.5	Focal lesions of the subendocardium of the apical and mid ventricular region with right ventricular involvement
2	Focal lesions extending over a wider area of both ventricles
2.5	Focal lesions extending over a wider area of both ventricles, extensive inflammatory cell infiltration, interstitial edema, rupture of myofibers
3	Confluent lesions of the apex, mid-left ventricle and right ventricle, extensive inflammatory cell infiltration, profuse edema
4	Confluent lesions throughout the heart

ISO indicates isoproterenol.

Table 2. Grading System Used to Score the Collagen Deposition in the ISO-Treated Animals

Score	Collagen Deposition
0	No damage
0.5	Few scattered collagen fibers throughout the heart
1	Patchy fibrosis in 1 to 5 areas (<20% of the field)
2	Patchy fibrosis in more than 5 areas (>20% of the field)
3	Contiguous subendocardial fibrosis of less than half the circumference
4	Contiguous subendocardial fibrosis of more than half the circumference
5	Transmural fibrosis of less than half the circumference
6	Transmural fibrosis of more than half the circumference
7	Total transmural fibrosis

ISO indicates isoproterenol.

that survived 24 hours after the last dose of ISO were combined in the ISO-treated group.

Survival

Male Sprague-Dawley rats were treated with either single or 2 doses of ISO ranging from 25 to 600 mg/kg. Out of the total 69 ISO-treated rats, 40 survived (58.0%) and 29 died (42.0%) within 24 hours of the last dose of ISO (Table 3). Out of total 29 deaths after ISO treatment, 16 rats died after first dose of ISO and another 13 rats died after the second dose of ISO.

Akinesia

ISO-treated rats showed varying degree of akinesis of the long-axis circumference of the left ventricle when the echo was recorded at 24 hours after the last dose of ISO injection. Using tracings of two-dimensional echocardiography (2D
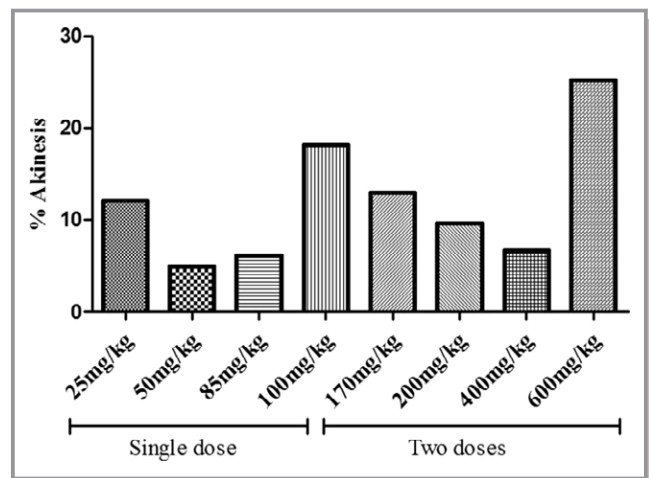

Figure 1. Flow chart depicting number of animals in each group in Protocol 1. ISO indicates isoproterenol.

Figure 2. Graphical representation of the different doses and dosing schedule used to induce apical akinesis in the rats. The number of animals with apical akinesis that survived 24 hours after isoproterenol subcutaneous injection was: Single-dose schedule-ISO 25 mg/kg (n=3); 50 mg/kg (n=1); 85 mg/kg (n=3); 100 mg/kg (n=5); Total dose of ISO administered using twice dosing schedule, in which half of each dose was injected at the interval of 24 hours: 170 mg/kg (n=8); 200 mg/kg (n=8); 400 mg/kg (n=1) and 600 mg/kg (n=1). ISO indicates isoproterenol.

Echo), total percentage of akinesis of the LV long-axis subendocardial circumference was calculated. Out of the total of 40 ISO-treated animals that survived 24 hours after injection, 30 animals showed apical akinesis and the remaining 10 animals did not demonstrate akinesis. Thus, 75% of surviving rats showed some degree of akinesis (See movies S1 and S2). The average percentage of akinesis was 12.1±1.6% in the ISO-treated rats with akinetic apical regions. To elucidate the effect of percentage of akinesis on apical fractional shortening and hemodynamics, we segregated the animals with small and large akinetic regions. We chose 10% akinesis as a cut-off as it is almost equivalent to
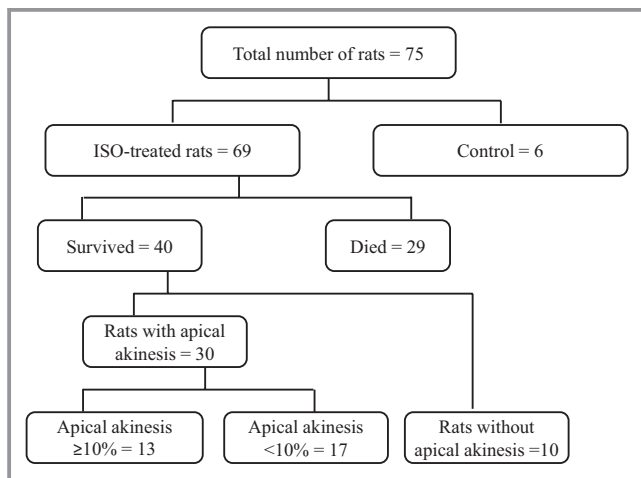
Table 3. Tabular Representation of the Number of Animals Used in the Protocol 1

Total Number of Animals	75
Control	6
Total ISO-treated rats	69
Survived	40 (58.0%)
ISO-treated subgroups	
Akinesis (≥10%)	13 (32.5%)
Akinesis (<10%)	17 (42.5%)
No akinesis	10 (25.0%)
Died	29 (42.0%)

ISO indicates isoproterenol.

the absolute mean of percent akinesis i.e., 12.1±1.6% observed after ISO administration. ISO-treated rats were divided into 3 subgroups on the basis of percent akinesis: 13 animals out of 40 survivors in the akinesis ≥10% subgroup (32.5%), 17 in the akinesis <10% subgroup (42.5%) and 10 in the No-akinesis subgroup (25%). The average percentage of akinesis of the LV circumference was 20.4±1.8% in the akinesis ≥10% subgroup and 5.7±0.5% in the akinesis <10% subgroup.

Fractional shortening

In the control (no ISO) group, apical fractional shortening (FS) was 46.7±4.2%; in the ISO-treated rats it was 37.8±4.6%. Apical FS was reduced to 16.5±3% in the akinesis ≥10% subgroup compared with the control group (*P*<0.05), whereas it was 36.4±6.8% in the akinesis <10% and 68.1±6.4% in the No-akinesis subgroup; the latter 2 did not differ significantly compared with the control group. The basal FS in the ISO-treated rats was 57.3±2.4%, which was comparable with the control (no ISO) group (54.8±5.9%) (Figure 3A and 3B).

Hemodynamics

Twenty-four hours after the last dose of ISO injection, elevated heart rate was observed in the ISO-treated rats versus control group and significant (*P*<0.05) increase was observed in the akinesis ≥10% subgroup. Mean arterial pressure was significantly (*P*<0.001) reduced to 67.3±2.1 mm Hg in the ISO-treated group as compared with 90.1±5.3 mm Hg in the control group. Left ventricular end-diastolic pressure did not differ in the ISO-treated group as compared with the control group whereas end-systolic pressure significantly (*P*<0.05) decreased in the ISO-treated group. The peak left ventricular pressure (+dP/dt) remained the same, but the peak negative (−dP/dt) decreased as compared with the control group. Significance (*P*<0.05) was achieved in the akinesis <10% subgroup only (Table 4).

Gross pathology

We did not observe gross confluent areas of myocardial infarction (TTC negative) as described in the coronary artery occlusion model.
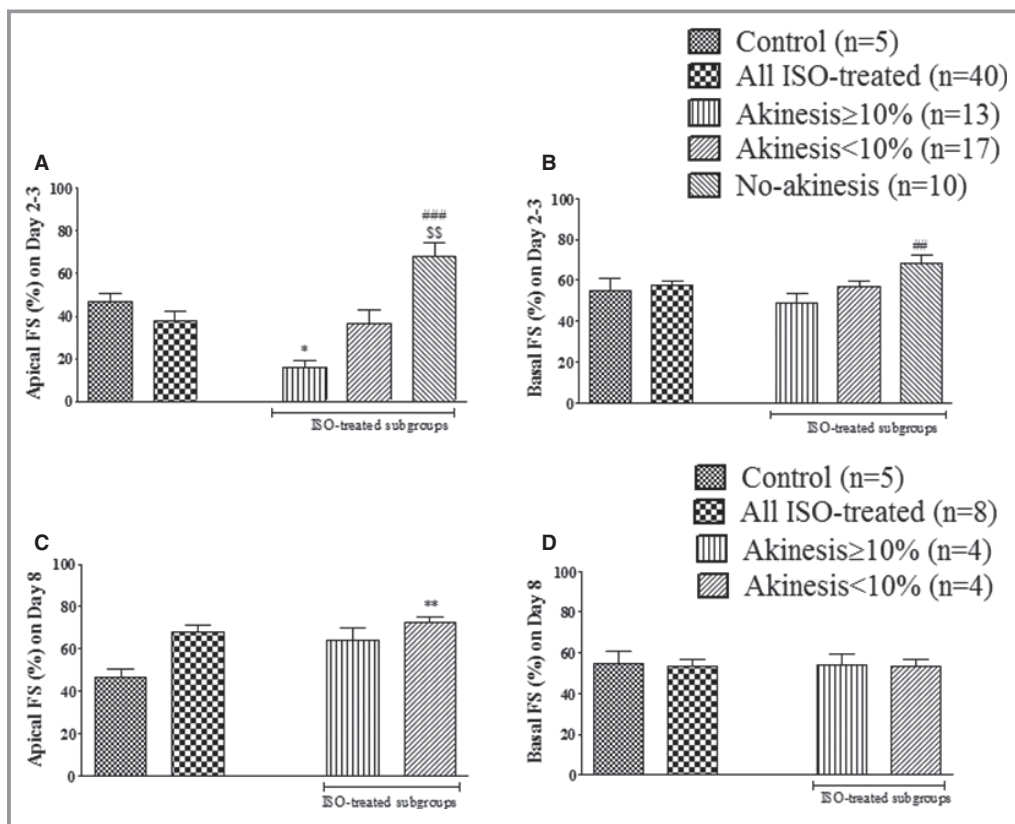

Figure 3. Bar graph representation of apical and basal fractional shortening (FS) on day 2 to 3 and day 8. Bars represent FS in the ISO-treated group and its sub-groups divided on the basis of percent akinesis of the long axis subendocardial circumference of the left ventricle (LV). A, Apical FS on day 2 to 3. B, Basal FS on day 2 to 3. C, Apical FS on day 8. D, Basal FS on day 8. Data are expressed as mean±SEM. **P<0.05, ***P<0.01 vs control; *##*P*<0.01, *###*P*<0.001 vs akinesis≥10% subgroup; *$$*P*<0.01 vs akinesis<10% subgroup. ISO indicates isoproterenol.

Table 4. Hemodynamic Changes on Day 2 to 3 and day 8 in the ISO-Treated Rats

	HR (Beats/min)	Pes (mm Hg)	Ped (mm Hg)	+dP/dt (mm Hg/s)	−dP/dt (mm Hg/s)	MAP (mm Hg)
Control (n=6)	255.8±19.00	110.0±8.6	7.1±1.2	4871.2±437.5	3970.8±445.8	90.1±5.3
Day 2 to 3						
ISO-treated (n=32)	310.3±11.7	85.7±2.8*	7.2±0.9	4400.9±176.1	2884.6±125.8**	67.3±2.1***
Akinesis≥10% (n=13)	344.4±25.9*	86.8±3.1	9.3±2.1	4332.6±219.2	3001.4±129.6	69.3±2.5*
Akinesis<10% (n=17)	295.7±18.7	86.2±5.9*	6.9±1.4	4059.4±277.5	2715.0±231.5*	63.9±3.3***
No-akinesis (n=10)	298.4±14.0	84.0±4.2*	5.7±1.2	4906.4±352.4	2999.9±247.4	70.1±4.8*
Day 8						
ISO-treated (n=8)	211.9±11.9	96.3±9.3	1.4±2.0	3712.0±339.9	2978.7±227.3*	69.0±6.6*
Akinesis≥10% (n=4)	185.9±10.0*	100.3±19.5	3.3±4.0	4009.5±550.5	3040.5±443.1	68.6±12.7
Akinesis<10% (n=4)	238.0±10.6	92.3±4.1	-0.5±0.5	3414.6±420.8	2916.8±205.3	69.4±6.4

Data are expressed as mean±SEM. +dP/dt indicates peak positive pressure; −dP/dt, peak negative pressure; HR, Heart rate; MAP, mean arterial pressure; Ped, left ventricular end-diastolic pressure; Pes, left ventricular end-systolic pressure.
*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs control.

Histopathology

Twenty-four hours after the last dose of ISO, histopathological analysis showed concomitant increase in the myocardial damage with increasing akinetic percentage. Semi-quantitative histopathologic score was higher at 2.0 [1.0] in the ISO-treated group versus 0.5 [0.4] in the control group. In the ISO-treated subgroups, histologic score was 2.0 [1.0] in the No-akinesis subgroup, 2.0 [1.5] in the akinesis <10% subgroup, and 3.0 [1.0] in the akinesis ≥10% subgroup (Table 5). All the ISO-treated rats showed scattered foci of necrosis, profuse interstitial edema, myocardial vacuolization, and infiltration of inflammatory cells (Figure 4). Monocytes and a few neutrophils were observed in the subendocardial region of both left and right ventricles. The myocardial injury spread from subendo-cardial to subepicardial myocardium with the increase in the

percentage of akinesis. The histological damage was mainly confined to the apical to mid-ventricular region of the heart, and rarely reached the base of the heart (Figure 4). A few scattered collagen fibers were also observed in the ISO-treated group (Figure 5B, Table 5). Intracellular lipid droplets were seen in the akinetic hearts (Figure 6).

Ultrastructural analysis

In animals that did not receive ISO, sarcomeres were arranged in a regular pattern surrounded by normal mitochondria with tightly packed cristae. Myocyte nuclei were normal, myofibrils were tightly placed alongside each other; and there was absence of wide interstitial space, lipid droplets, and vacuoles. In the ISO-treated rats, ultrastructural analysis showed extensive structural damage after 24 hours of ISO administration. However, there was presence of both normal and disrupted myofibrils with intramyofibrillar space. Most of the mitochondria were swollen with either filamen-tous or degenerated cristae with large rarefied matrix space, amorphous dense bodies and remnants of calcium phosphate deposits[3]; lipid droplets were aligned between the mitochon-dria. Large interstitial spaces were filled with edema and macrophages with engulfed degenerated cellular debris such as remnants of mitochondria and rough endoplasmic retic-ulum (Figure 7).

Table 5. Histological and Fibrotic Changes on Day 2 to 3 in the ISO-Treated Rats

	Histological Score	Fibrosis Score
Control (n=4)	0.5 [0.4]	0.0 [0.0]
ISO-treated (n=26)	2.0 [1.0]*	0.5 [0.5]
Akinesis≥10% (n=8)	3.0 [1.0]*	0.5 [0.8]
Akinesis<10% (n=11)	2.0 [1.5]*	0.0 [0.5]
No-akinesis (n=7)	2.0 [1.0]	0.5 [0.0]

Data are expressed as median [Interquartile range]. In the ISO-treated rats, on day 2 to 3, Mann−Whitney test used for fibrosis showed significance but the median semi-quantitative score of 0.5 was considered normal. Out of 40 ISO-treated rats, 32 were sacrificed on day 2 to 3 and 8 were sacrificed on day 8. Out of those 32, 26 were used for H&E staining and 6 for Oil red O staining. In the akinesis ≥10% subgroup, out of 13 rats, 8 for H&E, 1 for Oil red O were sacrificed on day 2 to 3 and 4 were sacrificed on day 8. In the akinesis <10% subgroup, out of 17 rats, 11 for H&E, 2 for Oil red O were sacrificed on day 2 to 3 and 4 were sacrificed on day 8. In the No-akinesis subgroup, out of 10 rats, 7 for H&E, 3 for Oil red O were sacrificed on day 2 to 3. ISO indicates isoproterenol.
*$P<0.05$ vs control.

Structural and functional changes 1 week post-ISO

To determine the time course of recovery of wall motion abnormalities and whether it was affected by percentage of akinesis, 8 ISO-treated rats that showed apical akinesis on echo 24 hours after the last dose of ISO were monitored and sacrificed after 1 week. In the ISO-treated group, 4 rats of the akinesis ≥10% subgroup (larger akinetic LV circumference)
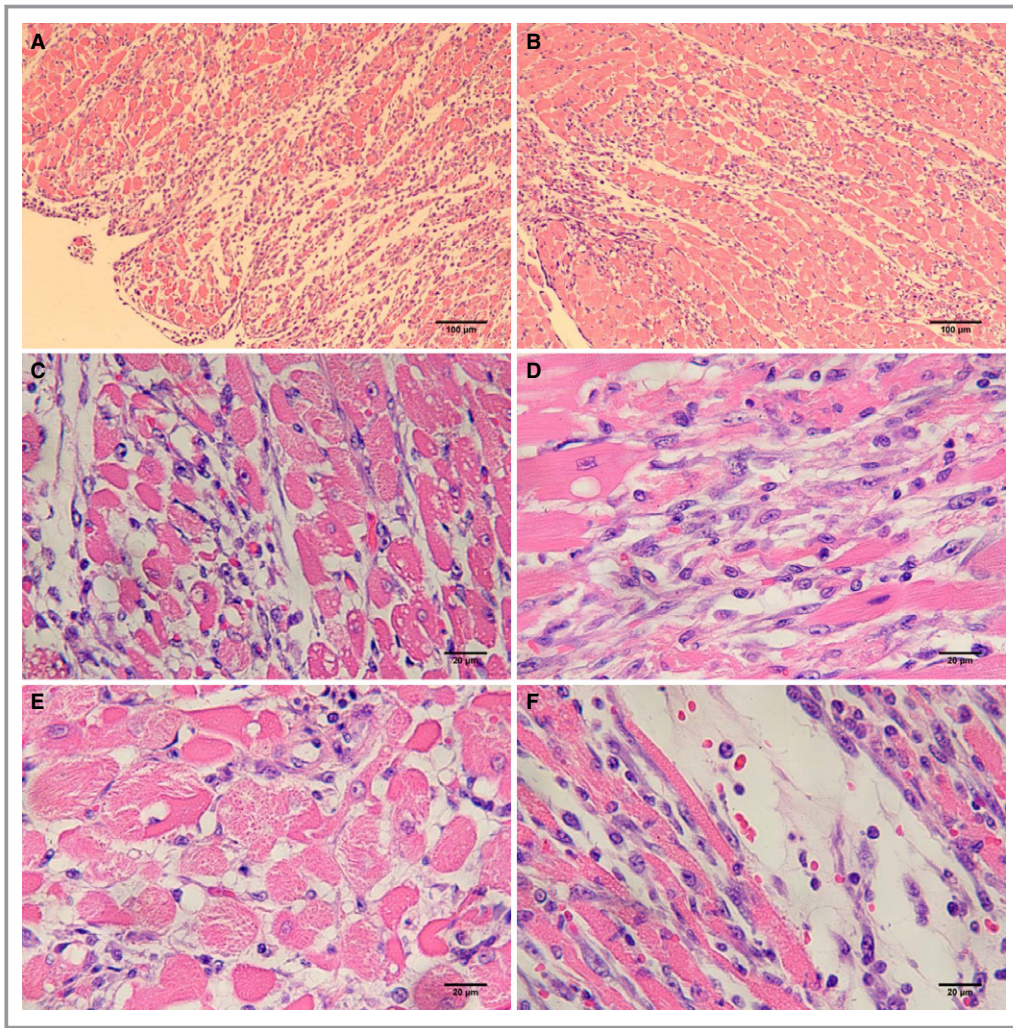
Figure 4. Representative photographs showing histopathological changes 24 hours after the last dose of isoproterenol. A, Extensive subendocardial necrosis. B, Foci of necrosis present over the entire thickness of the ventricular wall. C, Myocyte vacuolization. D, Macrophages infiltration in the necrotic region. E, Intramyofiber edema and vacuoles. F, Presence of mononuclear leukocytes and interstitial edema in the ISO-treated rat hearts. Original magnification ×10, Scale bar=100 µm; original magnification ×40, Scale bar=20 µm. ISO indicates isoproterenol.

and 4 of the akinesis <10% subgroup (smaller akinetic LV circumference) were included. One week post-ISO, echocardiography revealed normal wall motion in all of the ISO-treated rats irrespective of the percent akinesis at 24 hours. Noticeably, apical FS was markedly increased whereas the basal FS was similar as compared with the control group (Figure 3C and 3D). Hemodynamic parameters such as Pes, Ped, and +dP/dt were comparable with the control group, whereas, MAP and −dP/dt remained significantly decreased ($P<0.05$) at 1 week post-ISO. Heart rate was significantly depressed ($P<0.05$) in the akinesis ≥10% subgroup (Table 4). Despite the full recovery of the wall motion abnormality, histopathological analysis showed widespread necrosis, interstitial edema, inflammatory cells infiltration and vacuoles in the ISO-treated rats (Figure 8). The semi-quantitative score for picrosirius red staining was 3.5 [2.0] in the ISO-treated group which indicates marked collagen deposition (Figure 5C, Table 6).

Protocol 2

Survival

The number of surviving animals used for analysis of the data was 9 in each group (Figure 9). In the control group, rats were neither treated with ISO nor beta-blockers, and showed a 100% survival. In the ISO-treated group, initially 15 animals were recruited; 6 animals died after ISO administration. The
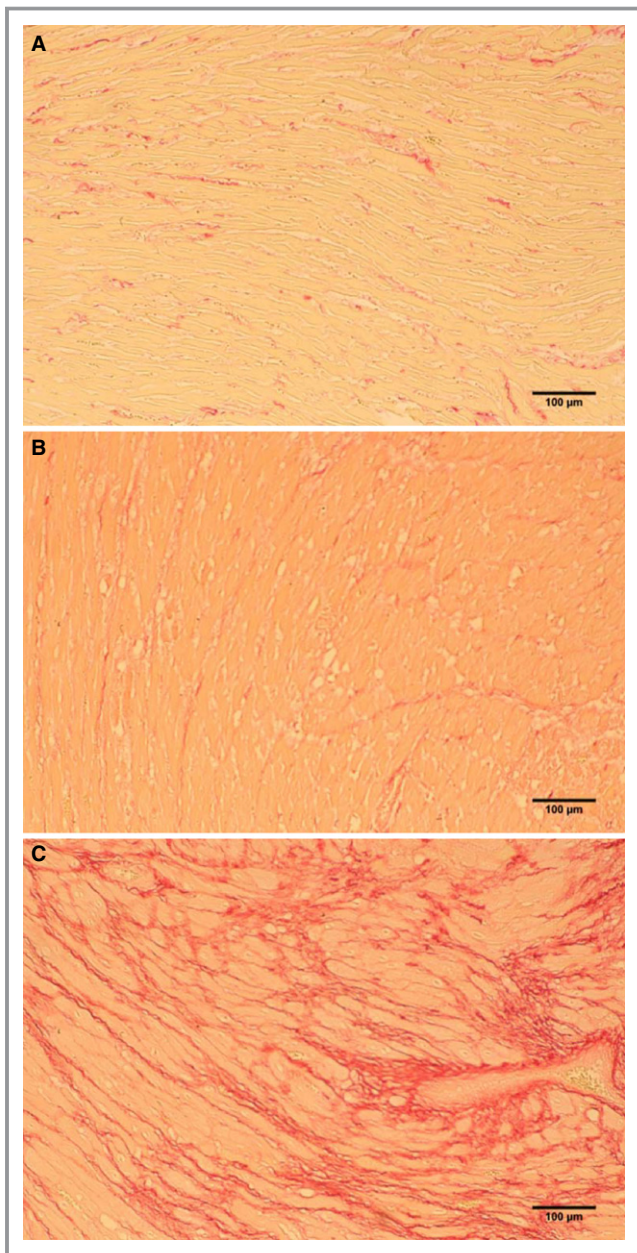
Figure 5. Representative photographs showing fibrosis on day 2 to 3 and day 8. Few scattered collagen fibers were present in the (A) control group and (B) 24 hours after the last dose of isoproterenol in the ISO-treated rats. (C) Extensive collagen deposition was seen on day 8 in the ISO-treated rat hearts. Original magnification ×10, Scale bar=100 μm. ISO indicates isoproterenol.

proportion of animals survived was 60%, similar to protocol 1. The percentage of rats that survived 24 hours post–ISO administration was 90% in the propranolol pretreated group and 100% in the metoprolol pretreated group. Although both beta-blockers, propanolol and metoprolol, increased survival after ISO injection, significance ($P<0.05$) was achieved only in the metoprolol pretreated group.
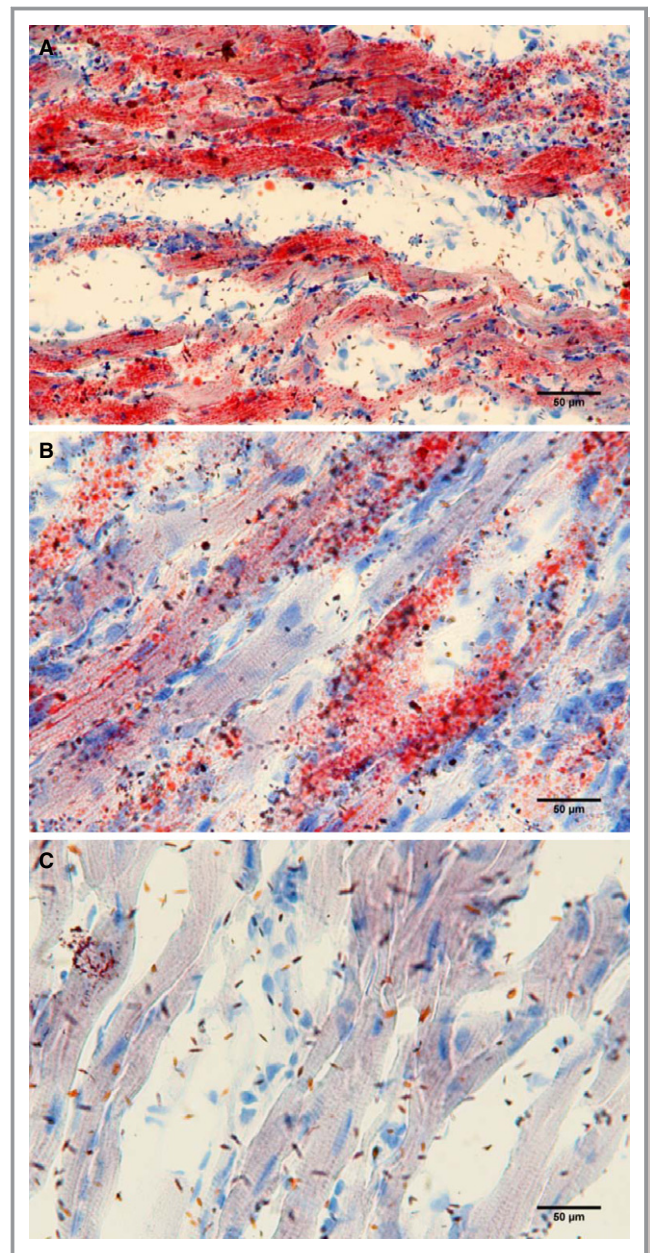

Figure 6. Photomicrograph showing lipid deposition (oil-red O positive) in the apical and mid-ventricular region 24 hours after the last dose of isoproterenol. A, Lipid deposition in the ISO-treated rat with 17.2% apical akinesis of the left ventricle. B, Lipid deposition in the ISO-treated rat with 8.2% apical akinesis of the left ventricle. C, Lipid droplets were absent in the ISO-treated rat with 3.6% apical akinesis of the left ventricle. In general, lipid deposition appeared more prominent in the hearts with higher percentage of akinetic LV circumference. Original magnification ×20, Scale bar=50 μm. ISO indicates isoproterenol.

Hemodynamics

Twenty-four hours after ISO administration, heart rate was significantly increased at 388.1±21.2 beats/min ($P<0.001$) in the ISO-treated group as compared with the control group
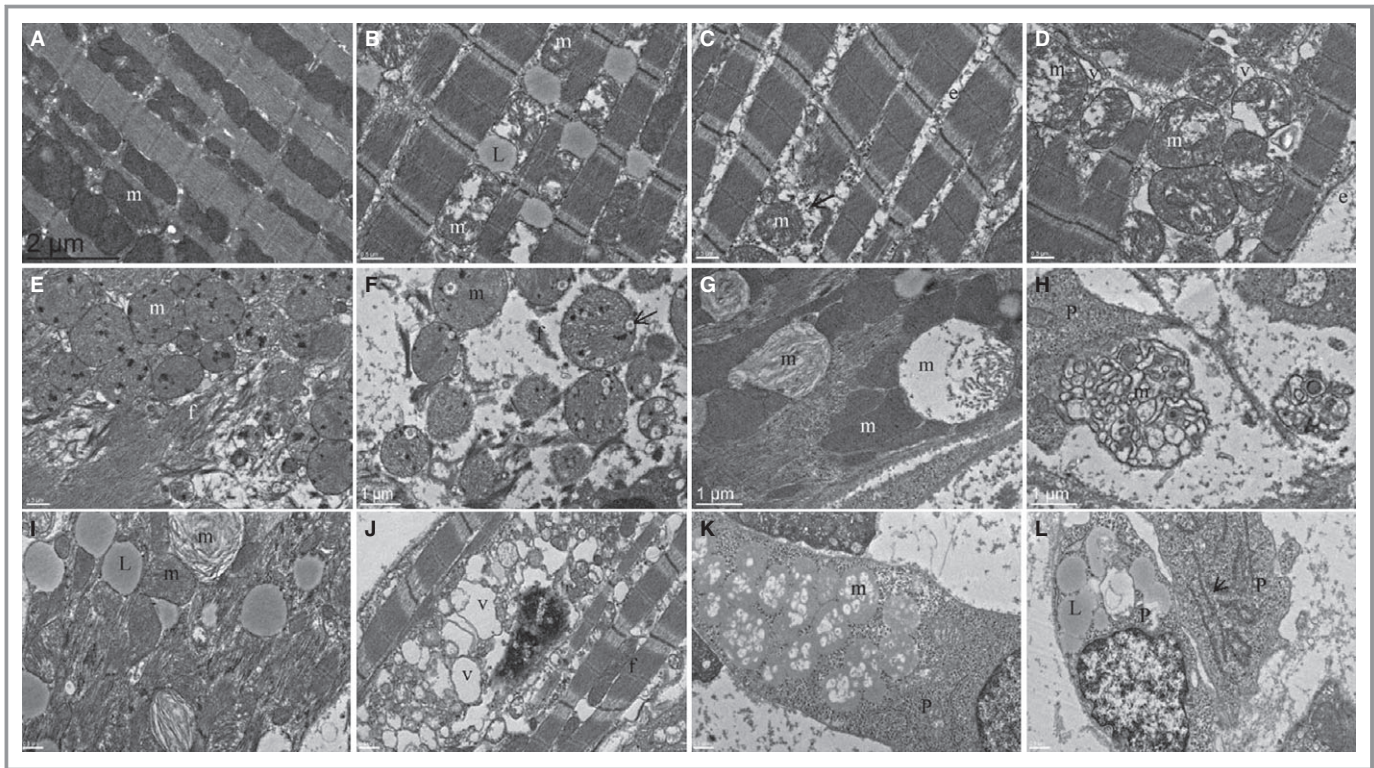
Figure 7. Ultrastructural changes in the apical akinetic myocardium 24 hours after ISO 100 mg/kg single subcutaneous injection. A, Normal sarcomeric architecture and mitochondria with tightly packed cristae (m) in the control group (Scale bar=2 μm); In the ISO-treated group, (B) organized sarcomeric structure, linearly aligned swollen mitochondria (m) interspersed with lipid droplets (L) along the myofibrils; (C) Intermyofibrillar edema (e), glycogen granule deposits (arrow); (D) Swollen mitochondria with large matrix space, vacuoles (v) and interstitial edema (e); (E) Disrupted myofibrillar (f) architecture interspersed with swollen mitochondria with amorphous dense bodies; (F) cluster of mitochondria having amorphous dense bodies and "halo" representing remnant of calcium phosphate deposits[3] surrounded by remnants of myofibrils (f); (G) degenerated mitochondria with filamentous remnants of cristae, swollen mitochondria with dissolved cristae and large matrix space; (H) Macrophage (P) engulfing the mitochondria with swollen cristae (m); (I) Filamentous cristal remnants in swollen mitochondria (m) and lipid droplets (L); (J) numerous vacuoles (v) present between the myofibrils (f); (K and L) Mitochondria (m), lipid droplets (L) and endoplasmic reticulum (arrow) engulfed by a large macrophage (P) surrounded by interstitial edema. (B through E, I through L) Scale bar=0.5 μm, (F through H) Scale bar=1 μm.

(265.9±12.2). Pretreatment with both propranolol and metoprolol 30 minutes prior to ISO injection showed slightly reduced heart rate even at 24 hours post-ISO, although the decrease was not statistically significant. There was no difference in other hemodynamic parameters such as MAP, Pes, Ped, +dP/dt, and −dP/dt between the ISO-treated and beta-blockers pretreated groups 24 hours post-ISO (Table 7).

Akinesis and fractional shortening

In the ISO-treated group, 3 of 9 animals showed apical akinesis which averaged 17.9±3.5% of the long axis LV circumference on echo; 4 of 9 animals showed average akinesis of 17.1±1.2% in the propranolol pre-treated group; and 3 of 9 animals showed average akinesis of 14.1±4.4% in the metoprolol pre-treated group. The number of animals and the percentage of akinesis were comparable in the beta-blocker pre-treated groups and ISO-treated group. The apical FS did not significantly differ between the control group

(70.1±4.5%) and the ISO-treated group (64.9±9.8%). Pretreatment with propranolol (51.7±9.9%) and metoprolol (57.6±10.1%) slightly decreased the apical FS as compared to both ISO-treated group and control group. In contrast, overall basal FS significantly increased (*P*<0.05) in all the ISO-treated groups with or without beta-blockers (70.3±6.2% in the ISO-treated group, 71.0±3.9% in the propranolol pre-treated group and 68.7±5.3% (*P*=NS) in the metoprolol pre-treated group) as compared with the control group (50.3±3.2%).

Morphological analysis and histopathology

Gross pathology did not show the presence of gross confluent infarct in the heart sections. In contrast, histopathological examination revealed extensive myonecrosis, inflammatory cell infiltration, profuse edema, and vacuoles in the ISO-treated group, similar to the protocol 1. Neither propranolol nor metoprolol pre-treated groups attenuated the myocardial
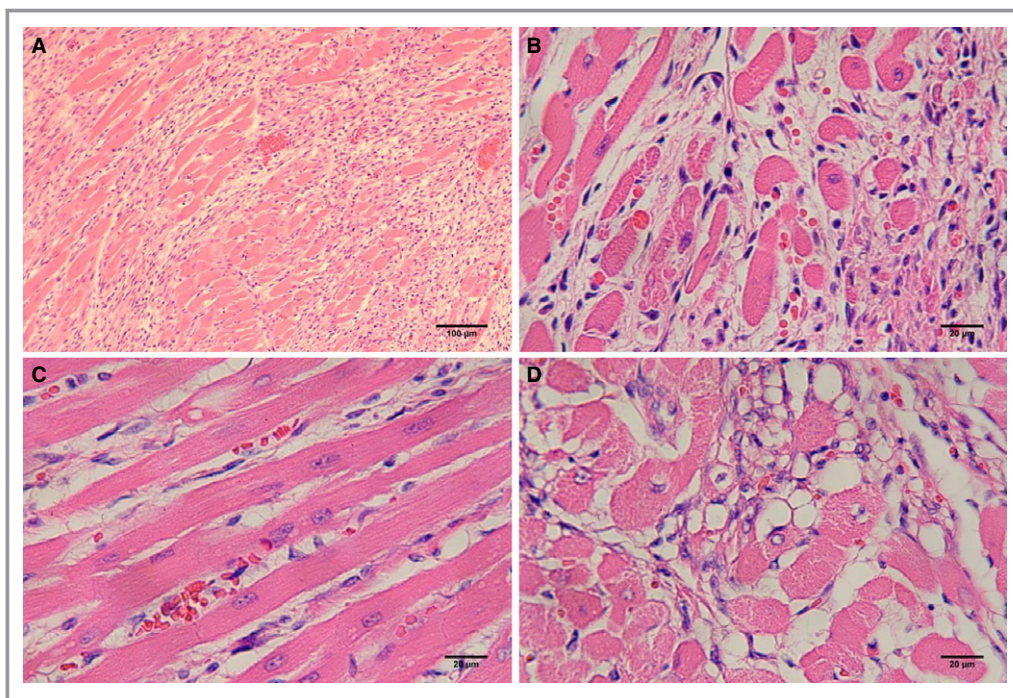
Figure 8. Representative photographs showing histopathological changes on day 8 (A) myonecrosis and interstitial space filled with collagen; (B) inflammatory cells and intramyofiber edema; (C) interstitial edema; (D) intramyofiber edema and marked vacuolization in the ISO-treated rat hearts. Original magnification ×10, Scale bar=100 μm; original magnification ×40, Scale bar=20 μm. ISO indicates isoproterenol.

damage as compared to the ISO-treated group. The semi-quantitive score given to assess myocardial damage was 2.5 [0.8] to the ISO-treated group, 2.0 [1.3] to the propranolol pre-treated group and 2.5 [1.0] to the metoprolol pre-treated group. Picrosirius red staining showed the presence of a few collagen fibers dispersed over the entire apical and mid-ventricular region of the myocardium in all the groups. As animals were sacrificed 24 hours after ISO injection, there was no extensive collagen deposition despite widespread myocardial damage (Table 8).

Discussion

Subcutaneous doses of ISO induced akinesis of the apical and mid-ventricular region of the left ventricle, which was

Table 6. Histological and Fibrotic Changes on Day 8 in the ISO-Treated Rats

	Histological Score	Fibrosis Score
Control (n=4)	0.5 [0.4]	0.0 [0.0]
ISO-treated (n=8)	2.5 [1.0]**	3.5 [2.0]**
Akinesis≥10% (n=4)	2.5 [1.8]*	4.0 [1.5]*
Akinesis<10% (n=4)	2.5 [1.8]*	2.5 [2.5]*

Data are expressed as median [Interquartile range]. ISO indicates isoproterenol. *$P<0.05$, **$P<0.01$ vs control.

congruous with the decrease in apical fractional shortening (FS); however, basal FS remained preserved. One week post-ISO administration, akinesis of the apical and mid-ventricular region was completely resolved; apical FS returned to normal values and hemodynamic variables normalized. Structural and ultrastructural analysis showed myonecrosis, mitochondrial damage, leukocyte infiltration (mainly monocytes), profuse interstitial edema, vacuoles and lipid deposition at 24 hours after the last dose of ISO and 1 week post-ISO. In addition to structural alteration, collagen deposition was also observed in the apical and mid-ventricular region 1 week post-ISO administration. Pretreatment with beta-blockers, propranolol and metoprolol, significantly increased the survival but did not

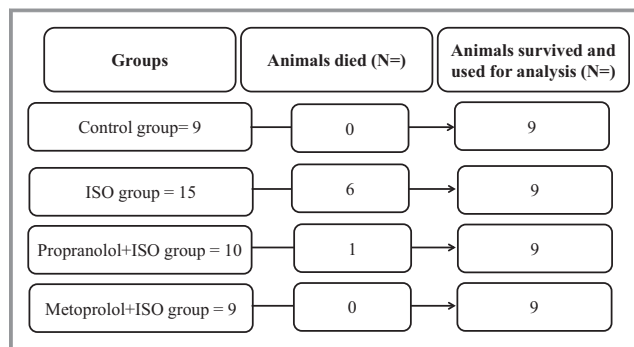| Groups	Animals died (N=)	Animals survived and used for analysis (N=)
Control group= 9	0	9
ISO group = 15	6	9
Propranolol+ISO group = 10	1	9
Metoprolol+ISO group = 9	0	9

Figure 9. Flow chart depicting number of animals in each group in Protocol 2. ISO indicates isoproterenol.

Table 7. Hemodynamic Changes 24 Hours After the ISO Administration in the β-Blocker Pretreated Rats

Day 2	HR (Beats/min)	Pes (mm Hg)	Ped (mm Hg)	dP/dt$_{max}$ (mm Hg/s)	dP/dt$_{min}$ (mm Hg/s)	MAP (mm Hg)
Control (n=9)	265.9±12.2	93.9±3.4	6.4±1.0	3995.7±307.7	3051.4±210.0	82.0±3.7
ISO 100 mg/kg (n=9)	388.1±21.2***	86.8±3.5	4.4±2.3	4086.2±413.3	2816.1±213.2	76.4±3.5
Propranolol (10 mg/kg)+ISO (n=9)	362.1±19.8**	92.0±2.7	7.8±1.8	3963.0±236.2	2686.8±123.4	79.4±3.6
Metoprolol (10 mg/kg)+ISO (n=9)	346.9±16.8*	90.1±2.0	8.5±1.6	4227.5±446.4	2921.5±215.4	75.5±2.8

Data are expressed as mean±SEM. HR, Heart rate; ISO, isoproterenol; Pes, left ventricular end-systolic pressure; Ped, left ventricular end-diastolic pressure; +dP/dt, peak positive pressure; -dP/dt, peak negative pressure; MAP, mean arterial pressure.
*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs Control.

decrease the incidence and degree of akinesis after ISO treatment.

Extent of Akinesis

We observed varying degree of apical akinesis in 75% of the total ISO-treated rats; whereas the remaining 25% showed similar structural damage but without contractile dysfunction. Also, change in apical fractional shortening was inversely proportional to the percentage of akinesis of the LV circumference. It is hypothesized that sudden increase in catecholamine levels induces Takotsubo cardiomyopathy in response to a stressful condition.[4–6] However, the total incidence of Takotsubo cardiomyopathy reported in the susceptible patients is 1 to 2.2%. This corroborates our observation that not all animals administered with high dose of catecholamine developed the characteristic wall motion abnormality.

Shao et al used a single intraperitoneal dose of ISO 50 mg/kg to induce Takotsubo cardiomyopathy, which induced an average apical akinesis of 35% of the long-axis LV circumference (somewhat larger than observed in our study). They observed complete recovery without any evidence of major histological changes and minimal fibrosis after 10 days.[7] In our study, maximum percentage of akinetic LV subendocardial circumference observed was 25% in the surviving rats irrespective of the ISO dose, i.e, there was no dose response for the degree of akinesis. However, the most distinguishing feature was the presence of marked

Table 8. Histological and Fibrotic Changes 24 Hours After the ISO Administration in the β-Blocker Pretreated Rats

Day 2	Histological Score	Fibrosis Score
Control (n=9)	0.5 [0.5]	0.5 [0.5]
ISO 100 mg/kg (n=9)	2.5 [0.8]***	0.0 [0.5]
Propranolol (10 mg/kg)+ISO (n=9)	2.0 [1.3] ***	0.0 [0.5]
Metoprolol (10 mg/kg)+ISO (n=9)	2.5 [1.0]**	0.0 [0.3]

Data are expressed as median [Interquartile range]. ISO indicates isoproterenol.
**$P<0.01$, ***$P<0.001$ vs Control.

histological changes such as foci of necrosis, myocardial vacuolization, inflammatory cell infiltration and profuse interstitial edema at 24 hours after the last dose of ISO administration. Moreover, histologic abnormalities remained persistent even after recovery of contractile dysfunction on day 8 along with collagen deposition in the previously akinetic region. We speculate that these histological differences might be due to different timing at which tissue was examined, staining used, and perhaps dose and route of isoproterenol administration used in the studies. Even though, recently published models of Takotsubo cardiomyopathy using epinephrine and isoproterenol are not in accord with our observation,[7,8] examination of patient subendocardial biopsies have shown evidence of structural damage.[9] Since we observed recovery of function at a time when histologic abnormalities were still prominent, we postulate that there is hypercontractility of non-damaged or lesser-damaged myocytes that compensate for the loss of function at the apex due to the severely damaged cells in the apical region.

Histological and Ultrastructural Features

Histology

In this study, histopathological analysis showed multiple foci of necrosis scattered among the normal myofibers, mononuclear leukocyte infiltration and profuse interstitial edema in the apical-midventricular region of both ventricles. Recent case reports and small clinical studies have confirmed the presence of these structural changes in the endocardial biopsy samples of the TC patients. In a recent case study, histopathological analysis of a myocardial biopsy sample obtained from a 71-year-old woman, who died 8 hours after the onset of TC, revealed multiple necrotic lesions, monocytes and neutrophil infiltration, and hemorrhage.[9] Also, in agreement with our observation, a clinical study conducted in 17 patients has correlated the cardiac magnetic resonance (CMR) images with the endomyocardial biopsy. The extent of interstitial edema corresponded with the akinetic region, irrespective of the coronary artery distribution. They found

that the severity of edema was directly proportional to the wall motion abnormality and low ejection fraction in patients. In accord with the previous clinical study, they also demonstrated presence of inflammatory (mainly monocytes) cells and disrupted myofibers in the biopsy samples.[10] A recent study done by Shao et al has shown that single intraperitoneal injection of ISO resulted in lipid accumulation in the akinetic region, which disappeared with the recovery of wall motion abnormality. They demonstrated that transporter protein ApoB, which exports surplus lipid from the cell, was downregulated in the ISO-treated akinetic mice. Furthermore, transgenic mice overexpressing ApoB lipoprotein showed 73.6% lower myocardial intracellular lipid and preserved cardiac function 2 hours post-ISO. Consistent with these findings, endomyocardial biopsies extracted from the affected apical region of 6 TC patients, showed excessive lipid accumulation in 4 patients with depressed contractile function and few intracellular lipid droplets in 2 recovered patients. These patients also demonstrated reduced expression of ApoB in the acute phase of TC with improved expression after recovery.[11] In agreement with the Shao et al findings obtained from the animal model and Takotsubo patients, we have also observed lipid deposition in the akinetic apical to mid ventricular region 24 hours after the last dose of ISO administration. Thus, our results also support the hypothesis that lipid deposition plays a role in the pathogenesis of Takotsubo cardiomyopathy.

Ultrastructural

We have for the first time demonstrated the ultrastructural changes in the akinetic region of the left ventricle 24 hours after ISO administration. We observed wide areas of sarcomeric disruption and disarray, which were interspersed with both normal and degenerating mitochondria. These degenerating mitochondria were either filled with amorphous densities and remnants of calcium phosphate deposits or empty space due to dissolved cristae. We speculate that the presence of these damaged intermyofibrillar mitochondria resulted in a decrease in ATP synthesis required for normal contraction, which, at least in part, plays a role in the development of akinesis. Similar alterations and mitochondrial fragmentation are typically observed in the infarct area of the ischemia-reperfused rat heart. However, in this model, we have few distinguishable mitochondrial abnormalities such as degenerating mitochondria with filamentous/lacey cristae, which are not typically seen in ischemia-reperfusion injury. Morphologic evidence of mitochondrial fission was also absent. In the widened and edematous interstitium, there were numerous large macrophages, which had engulfed degenerating nuclei, mitochondria, and rough endoplasmic reticulum. Few scattered collagen fibers were also seen alongside the myofibrils. We also observed lipid droplets predominantly aligned in between the mitochondria, which implies metabolic dysfunction along with structural damage and confirmed our findings by oil red O staining. These large lipid droplets were observed to be pressed against the myofibrils, which might be impeding normal contraction. Recent clinical and experimental studies have also corroborated the association between the wall motion abnormalities and cardiac metabolic dysfunction. In TC patients, integrated information from positron emission tomography (PET) and magnetic resonance imaging (MRI) scanning of the akinetic region, has revealed the transient shift from β-fatty acid (β-FA) oxidation to increased glucose metabolism resulting in free FA accumulation due to reduced uptake and utilization. Similar changes have been observed in the stunned myocardium, which strengthens the possibility that the viable stunned myocytes could be responsible for the wall motion abnormality.[12]

One week after ISO administration, histopathological damage was persistent along with collagen deposition in the apical-mid ventricular region of the ISO-treated rats. In contrast, wall motion abnormality was completely resolved in the previously akinetic rats. This recovery of contractile function might be due to recovery and probable hypercontraction of the previously stunned cardiomyocytes as a compensatory response to maintain the cardiac output. Similar regional dysfunction of viable myocardium is observed after reperfusion therapy in acute myocardial infarction patients. In ischemia-reperfusion injury, cardiomyocytes in the region supplied by the left coronary artery undergo either necrotic or apoptotic death and remain akinetic, whereas viable cardiomyocytes in the border zone, sometimes undergo stunning after revascularization and subsequently recover and compensate by hypercontraction. In contrast, in the rat model of Takotsubo cardiomyopathy, there are foci of surviving cardiomyocytes mixed with foci of necrotic cells, all within the apical region. Therefore, we hypothesize that some of these surviving cardiomyocytes might compensate for their dead neighbors by becoming hypercontractile, thereby restoring overall apical wall motion.

Beta-Blockers

Recent studies suggest that catecholamine induced contractile dysfunction occurs due to its action on the high density of β2 receptors predominantly present in the apical region of the heart.[8,13] Most cases of Takotsubo cardiomyopathy appear to involve the apex and, involve activation of β2 receptor mediated switch from Gs (stimulatory) to Gi (inhibitory) protein signaling resulting in negative inotropy at the apex. There are variant forms of Takotsubo cardiomyopathy that involve other regions of the ventricle and these forms might

implicate other β receptors. Both non-selective beta-blockers and β1 selective antagonist are also used to provide symptomatic treatment to Takotsubo patients. Recent experimental and case studies analyzed the effect of non-selective and β1 selective blocker treatment upon wall motion abnormality. Infusion of metoprolol (0.3 mg/kg/min) for 10 minutes to the cynomolgus monkeys, 48 hours after 2 repetitive epinephrine infusions (10 μg/kg/min), decreased myocytolysis and improved ejection fraction both at 10 minutes and at 24 hours after the infusion. Metoprolol also improved the expression of calcium handling and mitochondrial genes expression in the apical region 24 hours after infusion.[14] However, in another study, pretreatment with metoprolol 4 mg/kg could not attenuate the systolic dysfunction in-spite of reducing mean blood pressure and heart rate during and after immobilization induced Takotsubo cardiomyopathy in rats.[15] In a small clinical study, it has been shown that prior treatment with the therapeutic oral dose of beta-blockers is not sufficient to surmount the supraphysiologic levels of catecholamine and does not attenuate the severity and course of Takotsubo cardiomyopathy.[16] In agreement with this statement, we did not observe improved ejection fraction or reduced percentage of akinesis in the rats pretreated with a maximal dose of metoprolol (10 mg/kg). Moreover, the degree of myocardial damage was also comparable with the ISO-treated rats. Furthermore, it has been reported that intravenous injection of propranolol improves intraventricular pressure gradient, systolic blood pressure and ejection fraction in the Takotsubo patient with intraventricular obstruction.[17,18] In our study, pretreatment with a single intraperitoneal injection of propranolol failed to decrease the incidence and extent of akinesis, improve ejection fraction, or improve structural damage in the rats with wall motion abnormalities.

Nevertheless, we did observe improved survival with beta-blocker pretreatment; but the mechanism is not known. We hypothesize that acute mortality associated with sudden increase in catecholamine levels occurs due to malignant arrhythmias and beta-blockers increased survival by ablation of these lethal arrhythmias, although we did not specifically study this issue.

Conclusion

Our study has demonstrated that in a model of experimental TC characterized by transient apical but not basal wall motion abnormalities, myonecrosis, ultrastructural damage to mitochondria, lipid deposition and interstitial edema in the akinetic region are common features. Resolution of wall motion abnormality was not associated with attenuation of structural damage whereas there was significant collagen deposition in

the affected region. It is possible that the recovery of apical function was due to hypercontractility of viable cardiomyocytes in the apex. Prophylactic treatment with beta-blocker did not affect the development of akinesis but it has provided protection against the acute high mortality phase. Our study indicates that beta-receptor overstimulation is not solely responsible for contractile dysfunction; however, antagonism of beta-blockers might provide benefit in the patients by attenuating acute mortality.

Limitations of our study

As there was no mortality after 24 hours of ISO administration, the primary end points selected were echocardiography, hemodynamic and histologic assessment in the surviving rats after 24 hours of ISO injection. We did not assess any parameters before this time point; therefore, the cause of acute death in ISO-treated rats was not investigated. We cannot rule out the possibility that early death may have impacted our findings. For example it may be that those rats that died early would have had more extensive functional and histologic damage, had they been resuscitated and then survived 24 hours to 8 days. Unfortunately, many of these rats died suddenly and presumably due to arrhythmias. In the present study, we have focused on the pretreatment to determine whether beta-receptor antagonism can forestall apical akinesis. A study giving beta-blocker after initiating isoproterenol would be useful with more clinical relevant and should be considered in the future.

Disclosures

None.

References

1. Yatsinyuk BB. Hemodynamic disturbances and activation of lipid peroxidation in the pathogenesis of acute myocardial injury induced by propranolol in toxic doses. *Bull Exp Biol Med*. 2011;151:308–310.

2. Owczarek J, Jasińska M, Orszulak-Michalak D. Interaction between different doses of simvastatin after two-week administration and metoprolol injection on the heart rate in normocholesterolemic rats. *Acta Pol Pharm*. 2008;65:141–145.

3. Kloner RA, Ganote CE, Whalen DA, Jr, Jennings RB. Effect of a transient period of ischemia on myocardial cells. II. Fine structure during the first few minutes of reflow. *Am J Pathol*. 1974;74:399–422.

4. Ellison GM, Torella D, Karakikes I, Purushothaman S, Curcio A, Gasparri C, Indolfi C, Cable NT, Goldspink DF, Nadal-Ginard B. Acute beta-adrenergic overload produces myocyte damage through calcium leakage from the ryanodine receptor 2 but spares cardiac stem cells. *J Biol Chem*. 2007;282: 11397–11409.

5. Akashi YJ, Goldstein DS, Barbaro G, Ueyama T. Takotsubo cardiomyopathy: a new form of acute, reversible heart failure. *Circulation*. 2008;118:2754–2762.

6. Wittstein IS. Stress cardiomyopathy: a syndrome of catecholamine-mediated myocardial stunning? *Cell Mol Neurobiol*. 2012;32:847–857.

7. Shao Y, Redfors B, Scharin Täng M, Möllmann H, Troidl C, Szardien S, Hamm C, Nef H, Borén J, Omerovic E. Novel rat model reveals important roles of β-adrenoreceptors in stress-induced cardiomyopathy. *Int J Cardiol*. 2013;168: 1943–1950.

8. Paur H, Wright PT, Sikkel MB, Tranter MH, Mansfield C, O'Gara P, Stuckey DJ, Nikolaev VO, Diakonov I, Pannell L, Gong H, Sun H, Peters NS, Petrou M, Zheng Z, Gorelik J, Lyon AR, Harding SE. High levels of circulating epinephrine trigger apical cardiodepression in a β2-adrenergic receptor/Gi-dependent manner: a new model of Takotsubo cardiomyopathy. *Circulation*. 2012;126:697–706.

9. Elsokkari I, Cala A, Khan S, Hill A. Takotsubo cardiomyopathy: not always innocent or predictable. A unique post mortem insight. *Int J Cardiol*. 2013;167: e46–e48.

10. Iacucci I, Carbone I, Cannavale G, Conti B, Iampieri I, Rosati R, Sardella G, Frustaci A, Fedele F, Catalano C, Francone M. Myocardial oedema as the sole marker of acute injury in Takotsubo cardiomyopathy: a cardiovascular magnetic resonance (CMR) study. *Radiol Med*. 2013;118:1309–1323.

11. Shao Y, Redfors B, Ståhlman M, Täng MS, Miljanovic A, Möllmann H, Troidl C, Szardien S, Hamm C, Nef H, Borén J, Omerovic E. A mouse model reveals an important role for catecholamine-induced lipotoxicity in the pathogenesis of stress-induced cardiomyopathy. *Eur J Heart Fail*. 2013;15:9–22.

12. Ibrahim T, Nekolla SG, Langwieser N, Rischpler C, Groha P, Laugwitz KL, Schwaiger M. Simultaneous positron emission tomography/magnetic resonance imaging identifies sustained regional abnormalities in cardiac metabolism and function in stress-induced transient midventricular ballooning syndrome: a variant of Takotsubo cardiomyopathy. *Circulation*. 2012;126: e324–e326.

13. Mori H, Ishikawa S, Kojima S, Hayashi J, Watanabe Y, Hoffman JI, Okino H. Increased responsiveness of left ventricular apical myocardium to adrenergic stimuli. *Cardiovasc Res*. 1993 Feb;27:192–198.

14. Izumi Y, Okatani H, Shiota M, Nakao T, Ise R, Kito G, Miura K, Iwao H. Effects of metoprolol on epinephrine-induced takotsubo-like left ventricular dysfunction in non-human primates. *Hypertens Res*. 2009;32:339–346.

15. Ishikura F, Takano Y, Ueyama T. Acute effects of beta-blocker with intrinsic sympathomimetic activity on stress-induced cardiac dysfunction in rats. *J Cardiol*. 2012;60:470–474.

16. Palla AR, Dande AS, Petrini J, Wasserman HS, Warshofsky MK. Pretreatment with low-dose β-adrenergic antagonist therapy does not affect severity of Takotsubo cardiomyopathy. *Clin Cardiol*. 2012;35:478–481.

17. Kyuma M, Tsuchihashi K, Shinshi Y, Hase M, Nakata T, Ooiwa H, Abiru M, Hikita N, Adachi T, Shoji T, Fujise Y, Shimamoto K. Effect of intravenous propranolol on left ventricular apical ballooning without coronary artery stenosis (ampulla cardiomyopathy): three cases. *Circ J*. 2002;66:1181–1184.

18. Yoshioka T, Hashimoto A, Tsuchihashi K, Nagao K, Kyuma M, Ooiwa H, Nozawa A, Shimoshige S, Eguchi M, Wakabayashi T, Yuda S, Hase M, Nakata T, Shimamoto K. Clinical implications of midventricular obstruction and intravenous propranolol use in transient left ventricular apical ballooning (Tako-tsubo cardiomyopathy). *Am Heart J* 2008;155:526.e1–526.e7.