Short Term Disruption of Diurnal Rhythms Following Murine Myocardial Infarction Adversely Affects Long Term Myocardial Structure and Function

Faisal J. Alibhai¹, Elena V. Tsimakouridze¹, Nirmala Chinnappareddy¹, David C. Wright², Filio Billia⁴, M. Lynne O’Sullivan³, W. Glen Pyle¹, Michael J. Sole⁴, and Tami A. Martino¹

¹Cardiovascular Research Group, Department of Biomedical Sciences, University of Guelph, Ontario, Canada; ²Human Health and Nutritional Sciences, University of Guelph, Ontario, Canada; ³Department of Clinical Studies, University of Guelph, ⁴Division of Cardiology, Peter Munk Cardiovascular Centre, Toronto, Ontario, Canada.

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Address correspondence to:
Dr. Tami Martino
Cardiovascular Research Group
Biomedical Sciences
Room 4N-484
OVC Room 1646B
University of Guelph
Ontario, N1G2W1
Canada
tmartino@uoguelph.ca

Dr. Michael Sole
Division of Cardiology
Peter Munk Cardiovascular Centre
Toronto, Ontario, M5G2N2
Canada
mjssole@gmail.com

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ABSTRACT

Rationale: Patients in Intensive Care Units (ICU) are disconnected from their natural environment. Synchrony between environmental diurnal rhythms and intracellular circadian rhythms is essential for normal organ biology; disruption causes pathology. Whether disturbing rhythms post myocardial infarction (MI) exacerbates long-term myocardial dysfunction is not known.

Objective: Short term diurnal rhythm disruption immediately post-MI impairs remodeling and adversely affects long term cardiac structure and function in a murine model.

Methods and Results: Mice were infarcted by left anterior descending coronary artery ligation (MI model) within a 3-hour time window, randomized to either a normal diurnal or disrupted environment for 5 days, then maintained under normal diurnal conditions. Initial infarct size was identical. Short term diurnal disruption adversely impacted body metabolism and altered early innate immune responses. In the first 5 days, crucial for scar formation, there were significant differences in cardiac myeloperoxidase, cytokines, neutrophil and macrophage infiltration. Homozygous clock mutant mice exhibited altered infiltration post-MI, consistent with circadian mechanisms underlying innate immune responses crucial for scar formation. In the proliferative phase, 1 week post-MI, this led to significantly less blood vessel formation in the infarct region of disrupted mice; by day 14 echocardiography showed increased left ventricular dilation and infarct expansion. These differences continued to evolve with worse cardiac structure and function by 8 weeks post-MI.

Conclusions: Diurnal rhythm disruption immediately post-MI impaired healing and exacerbated maladaptive cardiac remodeling. These preclinical findings suggest that disrupted diurnal rhythms such as found in modern ICU environments may adversely affect long term patient outcome.

Keywords: Myocardial infarction, remodeling, circadian, innate immunity, intensive care, heart failure

Nonstandard Abbreviations and Acronyms:
ICU intensive care unit
L:D cycle light and dark cycle
MI myocardial infarction
MPO myeloperoxidase
ZT zeitgeber time
RER respiratory exchange ratio
INTRODUCTION

Over the years the management of patients in ICUs following MI has become increasingly intensive in an attempt to anticipate events and allow early intervention. These management strategies inadvertently increase noise, light and multiple other well-established stimuli in the ICU environment. This has resulted in a generally clinically unappreciated disruption of the endogenous circadian rhythms and sleep in acutely ill patients. Maintaining normal circadian rhythms is important as these are fundamental determinants of healthy cardiac physiology (for example, the cyclic variation in heart rate, blood pressure, and sympathovagal balance of the autonomic nervous system). Although circadian rhythms in timing of onset and tolerance to MIs are well established, the consequences of rhythm disturbance early post-MI have not been reported. The heart is relatively incapable of myocyte regeneration and early healing post-MI relies on coordinated removal of dead tissue through an early inflammatory phase, followed by replacement and remodeling of the myocardium and extracellular matrix. As remodeling progresses towards the maturation phase the heart changes size, shape and structure, and these processes can lead to ventricular dilatation, dysfunction and ultimately failure. Whether short term diurnal rhythm disruption post MI would impair the critical, orderly, temporal sequence of early myocardial repair and remodeling and adversely affect cardiac outcome is largely unknown.

To investigate the role of short term diurnal disruption on cardiac remodeling post-MI, we used a well-documented murine coronary artery ligation model and an established protocol for diurnal disruption, to simulate the ICU environment and its possible effects on the evolution of MI and ventricular remodeling in humans. Left anterior descending coronary artery ligation in mice was performed in a three hour time window (ZT01 – ZT04). Following MI the mice were monitored on their normal light (L) dark (D) cycle until lights out, then randomized to either continued normal L:D or a diurnal rhythm disrupted environment (10:10 LD). After only 5 days of normal versus disrupted environment, all groups were maintained in the normal L:D for up to 8 weeks post-MI. We demonstrate that the first few days post-MI are critical to long term tissue repair and remodeling, and that disrupting rhythms post-MI affects innate remodeling and adversely affects prognosis.

METHODS

Protocols are described in detail in online supplemental methods. All animal work was conducted under the guidelines of the Canadian Council on Animal Care. Briefly, 8 week old male C57Bl/6 mice (Charles River) were housed in a 12-hour light (L) and 12-hour dark (D) cycle (12:12 LD) for 2 weeks prior to surgery. Mice were subjected to the left anterior descending coronary artery ligation model (permanent occlusion without reperfusion) of myocardial infarction (MI). Sham animals underwent the same surgical procedures but without coronary artery ligation. All surgeries were performed between ZT01 – ZT04. Following MI the mice were monitored and maintained on their normal L:D cycle until lights out, then randomized to one of two groups. Either i) they continued to be maintained on their normal 12:12 LD environment, or ii) were placed in rhythm disrupted environment (10:10 LD). After only 5 days of normal versus disrupted environment, all groups were maintained in the normal 12:12 LD environment for up to 8 weeks post-MI. For determination of infarct size at 1 day post-MI, one set of hearts were collected and Evans Blue and 2, 3, 5-triphenyltetrazolium chloride (TTC) staining were performed. Hearts from a separate set of mice were collected for quantification of myeloperoxidase (a marker of early neutrophil response) as a measure of early innate immune response at 1, 2, 3 and 5 days post-MI. A third set of mice were used for immunohistochemistry for cardiac neutrophils (days 1, 2, 5), macrophages (days 3, 7), CD31-positive blood vessels (day 7), and for histologic analyses using Masson’s Trichrome stain (day 14). Plasma
from these mice was collected for measuring cytokines and corticosterone levels post-MI. A fourth set of hearts were collected for RTPCR of inflammatory markers and core clock genes at 36h and 48h post-MI. A fifth set were collected for pathophysiology including Comprehensive Lab Animal Monitoring System (CLAMS, days 4-6), and echocardiography (days 1, 3, 5, 7, 14). The sixth set were used for pathophysiologic assessments at 8 weeks post-MI, including echocardiography, hemodynamics, myofilament function and morphometric measurements. The seventh set were ClockΔ19 mice (i.e. these are ClockΔ19/ ClockΔ19 mice, homozygous for the CLOCK point mutation) bred on a C57BL/6 background with founders kindly supplied by Dr. Erik Herzog (Washington University) and Joseph S. Takahashi (University of Texas Southwestern). These and wildtype (WT) littermate controls were used to investigate the link between circadian disturbance and the early inflammatory phase of infarct healing. All endpoints assessed and n-values are provided in detail in Supplemental Table IX.

RESULTS

We found, in a murine model of left anterior descending coronary artery ligation (MI model), that diurnal disruptions in the first 5 days post-MI altered LV remodeling, worsened functional outcomes and accelerated progression towards heart failure. Table 1 shows that, compared with the normal-MI, the disrupted-MI mice had significantly greater adverse remodeling with increased (p<0.0001) left ventricular (LV) internal systolic dimensions (LVIDs, 4.87 ± 0.20 mm vs. 3.83 ± 0.24 mm) and internal diastolic dimensions (LVIDd, 6.06 ± 0.17 mm vs. 5.32 ± 0.18 mm), accompanied by decreased (p<0.0001) fractional shortening (FS) (19.72 ± 1.54% vs. 28.63 ± 2.68%) and decreased (p<0.0001) ejection fraction (EF) (45.23 ± 3.03% vs. 60.23 ± 4.36%) by 8 weeks post-MI. Consistent with heart size measurements and decreased function, the normal environment resulted in less adverse remodeling of LVIDs, LVIDd, %FS and %EF compared to mice that had been short-term in the disruptive environment post-MI.

Because the differences noted above could be correlated to cardiac hemodynamic responses, we next measured these in vivo (Table 1). As expected, the disrupted-MI mice showed significantly worsened cardiac profiles including decreased (p<0.0001) left ventricular (LV) end systolic pressure (LVESP, 82.40 ± 2.79 mmHg vs. 93.74 ± 1.82 mmHg), along with increased (p<0.002) end diastolic pressure (LVEDP, 6.17 ± 1.57 mmHg vs. 0.51 ± 1.00 mmHg). Consistent with the cardiac hemodynamic parameters, the disrupted-MI mice exhibited decreased (p<0.0001) dp/dt max (4848.41 ± 327.03 mmHg/s vs. 7018.01 ± 191.62 mmHg/s) and (p<0.0001) dp/dt min (4295.75 ± 316.72 mmHg/s vs. 6182.01 ± 284.14 mmHg/s). Significantly (p<0.0001) worse hemodynamic profiles were also observed in the short term disrupted-MI mice for left ventricular developed pressure (LDP), systolic blood pressure (SBP), and mean arterial pressure (MAP) than mice maintained in the normal environment throughout the study.

In view of the worsening functional profiles at 8 weeks post-MI, we explored whether these were associated with differences in structural remodeling. As expected, histological analyses revealed that the disrupted-MI mice had significantly greater cardiac hypertrophy by 8 weeks post-MI compared with the normal-MI mice as indicated by increased (p<0.0001) heart weight (HW) (8.19 ± 0.28 mg/g vs. 6.70 ± 0.31 mg/g; p<0.01) (Table 1, Figure 1A), and significantly (p<0.05) decreased infarct volume:LV mass ratio (7.77 +/- 0.68 %/mg vs. 9.99 +/- 0.34 %/mg (Figure 1B). Serial 1mm LV sections illustrate worse scar formation in disrupted-MI versus normal-MI hearts (Figure 1C) with initial infarct volumes similar in both groups (Figure 1D). Histologic analyses of serial LV sections stained with Masson’s trichrome (Figure 1E), and quantified, show that the disrupted-MI mice had greater (p<0.01) LV dilation (1917.61 ± 70.17 pixels vs. 1518.45 ± 47.68 pixels) (Figure 1F) and increased (p<0.01) infarct expansion (50.54 ± 1.72% vs. 42.61 ± 1.40%) (Figure 1G), as compared to normal-MI. These functional and structural data forecast a more rapid evolution to LV decompensation in the diurnal disrupted cohort.
The evolution of heart failure of a number of cardiovascular diseases may be associated with alterations in cardiac myofilaments (reviewed in 20, 21); we thus explored whether the disrupted-MI environment affected myofilament function by measuring cardiac actomyosin MgATPase activity. We found that myofilaments from disrupted-MI myocardium at 8 weeks exhibited reduced (p<0.05) maximal calcium-dependent myofilament ATP consumption (Table 1 and Supplemental Figure IA, IB, and Supplemental Table I). As might be expected from the functional data, mice maintained in the normal environment post-MI had an insignificant change in ATPase activity. To further investigate changes in cardiac myofilaments we examined phosphorylation of cardiac myofilament proteins by SDS-PAGE and Pro-Q Diamond stain. In the disrupted-MI group as compared to sham, troponin T (TnT) phosphorylation was decreased (p<0.05) by 17% in myofilaments, myosin binding protein C (MyBP-C) phosphorylation was decreased (p<0.05) by 25%, and troponin I was decreased (p<0.05) by 17% (Table 1 and Supplemental Figure IC, ID, IE, and Supplemental Table II). Consistent with the functional measurements at 8 weeks post-MI, the normal-MI hearts exhibited less change in myofilament protein phosphorylation than the hearts from mice initially housed in the disrupted-MI environment. Changes in myofilament phosphorylation status may be due in part to protein kinase A (PKA), as supported by the observed decrease in the PKA specific Serine 23/24 site of Troponin-I (Supplemental Figure IF, Supplemental Table III). These findings are consistent with the accelerated timeline to heart failure in the disrupted-MI mice.

To investigate the early mechanisms responsible, we examined the pathophysiology over the first 7 days. Initial infarct size was not significantly different (p>0.05) in mice at day 1 post-surgery, as expected, as measured by Evans Blue and TTC staining. As shown in Figure 2A, for animals in the disrupted environment at 1 day post-surgery, area at risk as a percentage of the left ventricle (AAR:LV) was 37.98 ± 2.01% vs. 38.20 ± 1.33%; infarct size as a percent of left ventricle mass (IN:LV) was 37.60 ± 1.87% vs. 37.83 ± 1.34%; and infarct size as a percentage of area at risk (IN:AAR) was 99.08 ± 0.47% vs. 99.05 ± 0.60%. One of the heart slices used for infarct size quantification is shown in Figure 2B. Furthermore, diurnal disruption did not affect early mortality in the animals used in this study; the incidence of cardiac rupture was 30% in normal-MI vs 32% in disrupted-MI, and these values were similar to reports by others (for example, Gao et. al.22). Once the animals were placed in the disrupted environment, whole body substrate utilization was altered (Figure 2C). The disrupted-MI mice had significantly different (p<0.05) RER during the light phase (0.95 ± 0.02 vs. 0.88 ± 0.01), and during the dark phase (p<0.05) (0.99 ± 0.01 vs. 0.93 ± 0.02) at day 5 post-MI, however, when the disrupted group was returned to the normal 24-hour environment (day 6) then whole body metabolism resynchronized as anticipated. Quantification of RER revealing altered substrate utilization in disrupted-MI is in Figure 2D. Consistent with the RER measurements, sham mice on the disrupted cycle also displayed altered daily substrate utilization that restored when they were returned to the normal 24 hour environment (Supplemental Figure IIA). Substrate utilization by days 5 and 6 was not affected by surgery, as anticipated (Supplemental Figure IIB). Metabolic parameters are in Supplemental Table IV. We next investigated whether disruption of the L:D cycle resulted in stress by measuring plasma corticosterone levels (Figure 2E). As anticipated, sham mice housed under normal L:D had a significant (p<0.05) corticosterone rhythm that peaked at the end of the light period and troughed in the dark. Similarly, sham mice in the disrupted environment had a corticosterone rhythm which, while slightly blunted, still peaked towards the end of their light cycle and declined through the dark. MI led to an overall increase (p<0.05) in corticosterone levels, similarly in both normal and disrupted groups, and they continued to display normal corticosterone responses to light similar to that observed in the sham groups. Even though the diurnal environment was only disrupted for 5 days, this significantly (p<0.05) exacerbated left ventricular diastolic remodeling as determined by echocardiography as early as 7 days post-MI (5.35 ± 0.12 mm vs. 4.94 ± 0.10 mm), and worsened (p<0.05) at day 14 (5.65 ± 0.15 mm vs. 5.22 ± 0.10 mm) (Figure 2F, Supplemental Table V), and persisted to 8 weeks post-MI (Table 1). Taken together, these reinforce the notion that disrupted diurnal environment alters whole body physiology and can act as a factor exacerbating early remodeling.
Further investigation of the early remodeling period in disrupted-MI revealed aberrant inflammatory mediator responses. As early as 36 hours post-MI, cytokine mRNA levels were disrupted in the heart (Figure 3A). Infarcted myocardium in the disrupted-MI mice displayed significantly (p<0.05) increased expression of interleukin-6 (il6) mRNA (25.80 ± 4.73 fold change vs. 8.11 ± 1.29 fold change), increased (p<0.05) monocyte chemoattractant protein 1 (mcp1/ccl2) mRNA (9.36 ± 1.03 fold change vs. 5.83 ± 1.13 fold change) and increased (p<0.05) monocyte chemoattractant protein 3 chemokine (mcp3/ccl7) mRNA (3.42 ± 0.48 fold change vs. 1.83 ± 0.47 fold change), as compared to normal-MI. Other cardiac mRNAs (tumor necrosis factor alpha, tnfa; interleukin 1 beta, il1b; matrix metalloproteinase 9, mmp9) were also elevated post-MI but did not differ (p>0.05) in disrupted-MI vs. normal-MI groups and thus were not pursued further (Figure 3A, Supplemental Table VI). Since il6 mRNA was increased (p<0.05) in disrupted-MI hearts (Figure 3A), we next looked at the blood cytokine levels (Figure 3B, Supplemental Table VII). In disrupted-MI mice at 36 hours, plasma IL6 levels were significantly increased (p<0.05) (3.64 fold change disrupted-MI vs. normal-MI; 17.90 ± 4.61 pg/ml vs. 4.92 ± 1.19 pg/ml) mice, and continued to rise over 60-hours (3.80 fold change disrupted-MI vs. normal-MI; 38.69 ± 10.43 pg/ml vs. 10.17 ± 2.35 pg/ml). As expected levels were not different between MI-groups prior to disruption (<24 hours), and normalized by 72-hours post-MI in both groups, and were not elevated in sham controls (Supplemental Table VII). Inflammatory mediators are critical signal molecules for infarct healing; altered elaboration may adversely affect scar formation.

To further investigate the early remodeling period, and in light of differences in chemokine mRNA expression involved in innate immune cell recruitment in disrupted-MI heart (mcp1/ccl2 and mcp3/ccl7, Figure 3A), we next investigated cardiac neutrophil recruitment by myeloperoxidase (MPO) assay. Figure 3C shows that, compared normal-MI, the disrupted-MI mice had an attenuated response with reduced (p<0.01) MPO activity in the heart at day 2 (50.79 ± 5.19 ng/mL/100mg tissue vs. 85.89 ± 10.76 ng/mL/100mg tissue) and prolonged elevated (p<0.01) MPO activity in the heart at day 5 (55.60 ± 2.39 ng/mL/100mg tissue vs. 26.56 ± 1.12 ng/mL/100mg tissue). Cardiac MPO levels were also different over the 5 day period by 2-way ANOVA, further supporting the notion of aberrant neutrophil recruitment in disrupted-MI heart (Supplemental Table VIII). Consistent with MPO as a measure of the innate inflammatory phase of LV remodeling post-MI, the sham mice had only minimal MPO activity in the heart and levels were not significantly different between the disrupted-sham and normal-sham groups (2 days; 18.24 ± 1.03 ng/mL/100mg vs. 16.85 ± 1.24 ng/mL/100mg tissue), (5 days; 9.58 ± 1.05 ng/mL/100mg tissue vs. 8.1 ± 0.43 ng/mL/100mg tissue) (Supplemental Table VIII). These data suggest that diurnal rhythm disruption contributes to alter cardiac remodeling by disrupting the innate post-MI cellular response.

To provide a more robust and definitive assessment of early inflammation stage we next measured neutrophil infiltration over the first 5 days by immunohistochemistry. As shown in Figure 3D, neutrophil infiltration into infarcted myocardium was reduced (p<0.05) by day 2 post-MI in disrupted hearts (158 ± 17 cells/mm² vs. 234 ± 22 cells/mm²). Overall levels of neutrophils leveled off in infarcted myocardium by day 5, consistent with the MPO response.. Some of the additional inflammatory cells in the early infarct region may be macrophages, as these cells infiltrate shortly after neutrophils. We observed elevated levels of macrophage infiltration in infarcted myocardium in disrupted-MI mice at day 3 (316 ± 11 cells/mm² vs. 215 ± 25 cells/mm²) and day 7 (115 ± 8 cells/mm² vs. 82 ± 6 cells/mm²) as compared to normal-MI infarcts (Figure 3E). Representative immunohistochemical stained sections are shown in Figure 4. There were fewer cells that stained positive for anti-neutrophil antibody at 2 days in disrupted-MI infarcts, even though there was increased overall cellular infiltration in the myocardium (Figure 4A). Moreover, there was enhanced staining for Mac3 macrophage antibody at 3 days in disrupted-MI infarcts (Figure 4B). Staining for Mac3 at day 7 was overall less than day 3, but staining remained stronger in disrupted infarcts (Figure 4C). Diurnal disruption altered innate immune cell recruitment into infarcted myocardium, underlying the exacerbated remodeling.
Infarct healing progresses with blood vessel formation in the infarcted region\textsuperscript{25}. Even though the disrupted mice had returned to the normal diurnal environment by day 5, there were subsequent effects on endothelial parameters by day 7. As shown in Figure 5A, there was less CD31+ blood vessels staining within the infarcted myocardium. Quantification across multiple sections confirmed significantly (p<0.01) decreased blood vessel density in disrupted-MI infarcts as compared to normal-MI infarcts (50 ± 4 vessels/0.1mm\(^2\) vs. 75 ± 3 vessels/0.1mm\(^2\)) (Figure 5B), and rich vessel staining in non-infarcted myocardium (Supplemental Figure III). Subsequent infarct expansion was assessed by Masson’s Trichrome stain at day 14 post-MI (Figure 5C). Masson’s Trichrome staining of extracellular matrix in the infarct region was also used as an indicator for potential defects in fibroblast activation (Figure 5D). Quantification of serial left ventricular (LV) sections revealed significantly (p<0.05) increased scar length in hearts from mice that had previously been in the disrupted environment as compared to the normal MI group (45.94 ±3.51 % of LV circumference vs. 34.69 ± 3.91 % of LV circumference) (Figure 5E, top). Scars were thinner in the disrupted-MI infarct region compared to normal-MI (26.22 ± 1.41 pixels vs. 37.85 ± 2.94 pixels), with infarct thickness determined by measuring at least 5 equidistant points across the scar region (Figure 5E, bottom).

In an effort to investigate the link between the circadian disturbance and dysregulation of the innate remodeling processes, we first performed realtime PCR for core circadian genes. The diurnal disrupted mice exhibited a loss of mRNA rhythms for circadian mechanism genes \textit{clock}, \textit{period2} (\textit{per2}), in both the infarcted (Figure 5F) and non-infarcted region (Supplemental Figure IV), whereas the mice housed in the normal environment maintained diurnal gene rhythms as anticipated. Importantly, since it has recently been shown that circadian mechanism modulates inflammatory responses via \textit{rev-erba}\textsuperscript{26,27} and \textit{nfil3}\textsuperscript{28,29}, and that circadian disruption dysregulates inflammatory responses\textsuperscript{30}, \textit{and since rtqcr data showed a change in rev-erba and nfil3 expression in disrupted hearts} (Figure 5F), we next investigated whether a mutation in the circadian mechanism altered inflammatory responses involved in infarct healing. Homozygous Clock\textDelta\textsubscript{19}/Clock\textDelta\textsubscript{19} mice had increased (p<0.05) neutrophil infiltration into infarcted myocardium, as compared to WT littermate controls (120 ± 9 cells/mm\(^2\) vs. 84 ± 9 cells/mm\(^2\)) (Figure 5G and 5H top). Additionally, homozygous Clock\Delta\textsubscript{19}/Clock\Delta\textsubscript{19} had increased (p<0.05) macrophage infiltration into infarcted myocardium compared to WT controls (383 ± 18 cells/mm\(^2\) vs. 261 ± 21 cells/mm\(^2\)) (Figure 5G and 5H, bottom). Collectively these are consistent with the notion that the circadian mechanism plays a role in modulating inflammatory responses crucial for early scar formation, and that disruption alters these responses. As summarized in Figure 5I, diurnal disruption affects the early inflammatory stage of infarct healing (phase 1), and even after the environment is restored the adverse effects continue to impact on the proliferative period (phase 2), and cumulatively worsen scar maturation (phase 3) and ultimately cardiac pathophysiology post-MI.

\textbf{DISCUSSION}

In this study, short term disruption of diurnal rhythms was used to test the hypothesis that maintaining normal rhythms is essential for cardiac repair and renewal post-MI, and that disturbing rhythms can have devastating effects on remodeling and outcome. In contrast, maintaining normal rhythms throughout the course of the disease better preserved cardiac structure and function. Our results further show that the short term diurnal disruptions can interfere with the early inflammatory phase of LV remodeling, adversely impacting on the innate immune infiltration and adversely affecting scar formation. Altered plasma IL6 and cardiac IL6 mRNA expression in disrupted-MI mice as a proinflammatory indicator is consistent with our hypothesis, and supported by earlier studies showing that circadian rhythm disruption alters cytokines in-vivo\textsuperscript{30,31}. Similarly, we observed changes in cardiac \textit{mcp1/ccl2} and \textit{mcp3/ccl7} mRNA expression in disrupted-MI hearts; these chemokines play integral roles in immune cell recruitment to infarcted tissue\textsuperscript{32,33}. Consistent with these findings were changes in MPO between MI groups over the first 5-days, suggesting
that diurnal rhythm disruption post-MI alters the neutrophil response. MPO itself has also been shown to play role in cardiac remodeling post-MI\textsuperscript{24} and chronically elevated MPO levels are a predictor of poor patient outcome\textsuperscript{35}. Immunohistochemistry confirmed altered neutrophil infiltration profiles in the early remodeling period. Macrophage recruitment to infarcted myocardium, which occurs after neutrophils\textsuperscript{23, 36} also displayed altered temporal profiles in the inflammatory period. Diurnal rhythm disruptions can also impact fibroblast and endothelial cell function exacerbating scar formation and ultimately expansion. Moreover, the observed changes in expression of core circadian mechanism genes clock and per2, and their altered output genes reverba and nfil3 that regulate innate inflammatory cells, prompted us to examine what happens to immune cell recruitment when the clock is altered. To investigate this we used ClockΔ19/ClockΔ19 mice, which exhibited altered macrophage and neutrophil infiltration into infarcted myocardium. These findings were consistent with the notion that the circadian mechanism plays an important role underlying inflammatory recruitment key for infarct remodeling. There is a crucial order sequence of humoral and cellular factors in LV infarct remodeling. In a corollary to our experiments, early innate inflammatory pathways obligatorily favor survival of myocardium in the border zone during the acute period post-MI\textsuperscript{37-39}. Therefore diurnal rhythm disruption has the ability to alter cardiac remodeling through innate immune response disruption post-MI.

Even though the animals were returned to the normal diurnal cycle after just 5 days, the adverse remodeling led to long term effects on myofilament function and reduced calcium sensitivity consistent with the development of heart failure by 8 weeks post-MI. Decreased ATPase activity at saturating levels of free calcium suggest differences in myofilament modifications between disrupted-MI and normal-MI myocardium. We also examined phosphorylation associated with myofilament function in cardiac remodeling\textsuperscript{40-42}, possibly mediated through protein kinase A. Intracellular pathways for sarcomere assembly and myocardial growth and renewal are clock dependent\textsuperscript{43, 44}; these could certainly be impaired by circadian disruption. Many additional factors subject to diurnal regulation undoubtedly conspire to yield the adverse consequences of diurnal disruption post-MI. For example cardiac loading would contribute to increased infarct expansion, increased early loading and side-to-side cell slippage – potential precursors to long term maladaptive ventricular topography\textsuperscript{45, 46}.

The mechanism of the pathology lies in the disruption of the regulation of innate immune responses underlying normal biology. Most recently, studies have demonstrated that the core circadian clock modulates macrophage activation and normal inflammatory function in mice\textsuperscript{26, 27}. Moreover, circadian dysynchrony in mice alters core clock gene rhythms in immune tissues (spleen, thymus, peritoneal macrophages), and enhances blood cytokine levels in response to LPS challenge\textsuperscript{30}. Collectively these studies support the notion that the circadian mechanism regulates inflammatory responses, and are consistent with our findings that diurnal disruption alters the inflammatory responses crucial for infarct healing. The disturbed diurnal environment disrupts the orderly temporal sequence of events critical to an efficient repair process (Figure 5I). In phase 1 this would disrupt neutrophil and macrophage infiltration, and production of inflammatory mediators such as MPO and cytokines. In early stage 2 when the diurnal environment is still disrupted this can affect endothelial cell and fibroblast cell function. There is disruption of circadian rhythms within the cells themselves. Approximately 13% of myocardial genes exhibit day/night variation\textsuperscript{47}; high proportions of these are associated with metabolism, signaling, and repair. Even once the normal environment is restored, this can have repercussive effects on infarct expansion, scar thickness and maturation, and ultimately organ pathophysiology. Future studies using circadian genetic mutant mice to explore inflammation in post-infarction remodeling are worthy of investigation.

Though not yet generally appreciated, failure to entrain to an L:D cycle not in close synchrony with the intrinsic cellular circadian period inhibits organ renewal and repair resulting in organ pathology. Cell physiology is 4-dimensional: the substrate and enzymatic components of a given biochemical pathway must be available not only in the right compartmental space within the cell but at the right time. We previously showed in our experimental animal studies that circadian dysynchrony exacerbates heart disease in the
TAC model of pressure overload induced cardiac hypertrophy. A second study revealed that in +/tau hamsters, which exhibit dyssynchrony between the 24 hour day and their intrinsic shortened circadian period of 22h, profound dilated cardiomyopathy developed along with renal disease. Similar deleterious effects on survival are seen in cardiomyopathic hamsters subjected to chronic shifts in the light:dark cycle. In humans, disturbing rhythms such as in shift workers increases the risk of adverse cardiac events such as myocardial infarction and sudden death. Thus an important mechanism for our observations is disruption of the appropriate temporal sequence of innate immune and intrinsic cellular responses to the myocardial insult by the adverse diurnal environment.

In these experiments we used a murine model of permanent myocardial infarction. Unlike human patients the murine subjects are healthy, young, homogenous, free of coexisting illness and receiving no other medications. The pattern of cellular and inflammatory responses to the infarct (although somewhat accelerated) reflects that found in larger animals including humans. There is a circadian variation of infarct size in the murine model which predicted a similar circadian variation in patients. Experimental observations in rat and mouse infarct models have provided the impetus for clinical trials – successfully predicting the pathogenesis and pharmacological prevention of heart failure in post myocardial infarction patients. Unlike our model, the majority of human patients reaching hospitals in a timely fashion in the Western World will undergo reperfusion, however, many do not - their presentation to hospital is too late for myocardial salvage or their procedure is unsuccessful, exhibiting incomplete revascularization or no reflow. Regardless, the reperfused myocardium should also exhibit an orderly cascade of molecular and cellular responses; these also should be susceptible to diurnal disruption similar to that demonstrated here.

Finally, no animal model completely reflects patient experience in the ICU. The accepted model of diurnal disruption we used is actually quite benign and stress-free compared to that experienced by patients post-infarction. Modern ICU care has disconnected patients from their natural diurnal environment. Noise, light, nursing and medical procedures, pharmacology, ventilators and discomfort and pain conspire to deprive the patient of sleep and further the alterations of heart rate, blood pressure and neurohormonal secretion occasioned by the primary event. This is not a necessary consequence of intensive monitoring and nursing care as each of the noxious stimuli in the ICU can be subject to some control. Our results suggest that diurnal disruption post-MI interferes with the early innate inflammatory phase of LV remodeling and, in the long term, adversely impacts on scar formation worsening functional outcome and accelerating cardiac surrogates of progression to heart failure.

Our observations are certainly not conclusive for human patients but at minimum they focus attention on the toxic diurnal environment of many of today’s ICUs. Since normal physiology caters to a diurnal environment, maintenance of normal diurnal rhythms during the recovery phase post-MI should aid in a coordinated and effective infarct healing response and improve patient outcome. More intensive monitoring and care is not necessarily better. We hope our preclinical study will stimulate the initiation of clinical trials reappraising the “diurnal health” not only of contemporary ICUs but also of present-day hospital environments.
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**FIGURE LEGENDS**

**Figure 1. Rhythm disruption immediately post-MI impaired healing and exacerbated maladaptive cardiac remodeling.** Mice in a disrupted environment for only 5 days post-MI and then returned to a normal environment for 8 weeks had **A** increased heart weight (HW):body weight (BW) (n=10/MI, n=5/sham) and **B** decreased infarct volume:LV mass ratio (n=5/group). **C** Representative images, worse scar formation and LV dilation (1mm sections) in disrupted-MI vs. normal-MI hearts. **D** Infarct volume (%LV) was initially the same (n=5/group). **E** Representative serial LV sections, worse scar formation in disrupted-MI vs the normal environment throughout. Quantification of Figure 1E, in **F** showing greater LV dilation, **G** increased infarct expansion in disrupted-MI hearts (n=5/MI, n=3/sham). Mean±SEM, *=p<0.01 disrupted-MI vs. all groups, ‡=p<0.001 normal-MI vs. sham.

**Figure 2. Diurnal rhythm alters pathophysiology over the first 7 days.** **A** Infarct size did not differ initially (1 day) post-MI confirming same techniques (n=6/group). Quantification determined by i) area at risk as % of LV (AAR:LV), ii) infarct area as % of LV (IN:LV), iii) infarct area as % of AAR (IN:AAR). **B** Representative images, infarct size quantification, 1 day post-MI, Evan’s blue and 2,3,5-triphenyltetrazolium chloride stain. **C** Whole body metabolic substrate utilization rhythms are affected by diurnal disruption (day 5), but restored once mice are returned to the normal diurnal environment (day 6), by respiratory exchange ratio (RER), **D** quantification of RER. n=5/group, *=p<0.05 disrupted-MI vs. normal-MI. Metabolic values are in Supplemental Table IV, Figure II. **E** Sham mice days 1-3, normal plasma corticosterone response to light (left). MI mice, overall increase in plasma corticosterone, normal response to light (right). Boxes, normal 12:12 L:D (n=5/MI, n=3/sham). **F** Short term diurnal disruption worsened LV internal dimensions at diastole (LVIDd) by day 7 post-MI (n=6/MI, n=3/sham). Pathophysiology, days 1-14 are in Supplemental Table V. *=p<0.05 disrupted-MI vs. normal-MI on the same day, one-way ANOVA Tukey post hoc.

**Figure 3. Rhythm disruption altered early innate inflammatory responses post-MI.** **A** Cardiac mRNA expression, *il-6, mcp-1/ccl2, mcp-3/ccl7,* was altered in disrupted-MI (black) vs. normal-MI (white) infarcted myocardium. Cardiac mRNA expression, *il1b, tnf-a mmp9* was elevated but not different between MI groups. *=p<0.05, n=5/group, values in Supplemental Table VI. **B** Plasma IL-6 was elevated in disrupted-MI mouse, *p<0.05, n=5/time/group, values in Supplemental Table VII. **C** Cardiac
myeloperoxidase (MPO, a measure of neutrophil infiltration) in normal-MI hearts was evident by day 1, peaked at day 2 then declined. In contrast, in disrupted-MI heart the response was attenuated by day 2, and prolonged by day 5. 2-way ANOVA, n=16/MI group, 4/time. n=12/sham, 3/time. Mean±SEM. *=p<0.05, values in Supplemental Table VIII. D) Neutrophil infiltration was reduced in infarcted myocardium of disrupted-MI (black) vs. normal-MI (white) hearts by day 2. E) macrophage infiltration increased days 3, 7 post-MI. *=p<0.05, mean ± SEM, n=3 sections/heart, 6 areas/section, 4 hearts/group.

**Figure 4. Immunohistochemistry of neutrophils and macrophages in infarcted myocardium.** Innate immune cells quantitated as cell counts/mm². Representative sections disrupted-MI infarcts have fewer A) neutrophils (day 2), B) more macrophages (day 3) and C) (day7), as compared to normal-MI. n=3 sections/heart/antibody, 6 areas/section, 4 hearts/MI and 3 hearts/sham group.

**Figure 5. Adverse remodeling persists into the proliferative phase.** A) Decreased CD31+ blood vessel staining (day7), disrupted-MI vs. normal-MI infarcts (non-infarcted in Supplemental Figure III), and B) quantification of vessel density per 0.1mm² of infarct tissue, day 7 post-MI. n=4/group. C) Infarct expansion is worse in disrupted-MI hearts (day14), representative serial LV sections (Masson’s Trichrome), and D) infarct thickness and extracellular matrix deposition a measure of myofibroblasts and scar formation, and E) quantification of % infarct expansion (top), and infarct thickness (pixels) with 5 measurements taken over equidistant points in scars (bottom). n=6 hearts/group. F) Loss of diurnal gene expression in the infarcted myocardium of diurnal disrupted mice, core circadian genes clock, per2 mRNA, and circadian regulated inflammatory modulators reverba, nfil3 mRNA. (n=5 hearts/time/group). G) Representative sections, ClockΔ19/ClockΔ19 mice, showing increased neutrophil (top) and macrophage (bottom) infiltration versus WT littermate controls, and H) quantification, day 3 post-MI, n=7/MI, n=3/sham. I) Proposed mechanism for cardiac remodeling post-MI. Early inflammatory cell recruitment to infarcted myocardium (phase 1), proliferation of endothelial cells and fibroblasts (phase 2), and scar maturation (phase 3) exhibit an orderly temporal sequence critical to efficient repair. Diurnal disruption alters these pathways exacerbating pathophysiology. Mean±SEM, *=p<0.05.
TABLE 1. Short Term Diurnal Disruption Post-MI Adversely Affects Long Term Cardiac Structure and Function

<table>
<thead>
<tr>
<th></th>
<th>Disrupted – MI</th>
<th>Normal – MI</th>
<th>SHAM</th>
<th>p value ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Morphometry</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW (g)</td>
<td>30.40 ± 0.44</td>
<td>31.01 ± 0.87</td>
<td>30.83 ± 0.81</td>
<td>0.8100</td>
</tr>
<tr>
<td>HW (mg)</td>
<td>248 ± 8.00 §</td>
<td>206 ± 8.00 **</td>
<td>136 ± 2.00</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HW:BW (mg/g)</td>
<td>8.19 ± 0.28 †</td>
<td>6.70 ± 0.31 **</td>
<td>4.42 ± 0.08</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HW:TL (mg/mm)</td>
<td>13.07 ± 0.69 †</td>
<td>10.18 ± 0.37 †</td>
<td>7.62 ± 0.06</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Maximal ATPase</td>
<td>196 ± 6.00 †</td>
<td>221 ± 7.00</td>
<td>213 ± 4.00</td>
<td>0.0162</td>
</tr>
<tr>
<td>Phospho-MyBPc (au)</td>
<td>1.33 ± 0.07 *</td>
<td>1.87 ± 0.07</td>
<td>1.76 ± 0.07</td>
<td>0.0007</td>
</tr>
<tr>
<td>Phospho-TnT (au)</td>
<td>1.92 ± 0.04 *</td>
<td>2.57 ± 0.06</td>
<td>2.32 ± 0.10</td>
<td>0.0020</td>
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<tr>
<td>Phospho-TnI (au)</td>
<td>2.23 ± 0.11 †</td>
<td>2.41 ± 0.14</td>
<td>2.69 ± 0.05</td>
<td>0.0044</td>
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<tr>
<td>Pathophysiology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td>559.89 ± 12.53</td>
<td>582.69 ± 18.16</td>
<td>580.45 ± 19.37</td>
<td>0.5424</td>
</tr>
<tr>
<td>LVIDd (mm)</td>
<td>6.06 ± 0.17 *</td>
<td>5.32 ± 0.18 **</td>
<td>4.15 ± 0.10</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LVIDs (mm)</td>
<td>4.87 ± 0.20 *</td>
<td>3.83 ± 0.24 †</td>
<td>2.29 ± 0.12</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>FS (%)</td>
<td>19.72 ± 1.54 †</td>
<td>28.63 ± 2.68 **</td>
<td>44.87 ± 1.83</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EF (%)</td>
<td>45.23 ± 3.03 †</td>
<td>60.23 ± 4.36 **</td>
<td>81.87 ± 1.78</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LVESP (mmHg)</td>
<td>82.40 ± 2.79 †</td>
<td>93.74 ± 1.82 †</td>
<td>103.51 ± 1.03</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>LVEDP (mmHg)</td>
<td>6.17 ± 1.57 †</td>
<td>0.51 ± 1.00</td>
<td>-1.36 ± 1.02</td>
<td>0.0022</td>
</tr>
<tr>
<td>LDP (mmHg)</td>
<td>76.17 ± 3.71 †</td>
<td>93.23 ± 1.76 †</td>
<td>105.36 ± 1.84</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>82.86 ± 1.53 †</td>
<td>91.75 ± 2.09 †</td>
<td>99.80 ± 1.05</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>48.91 ± 2.75</td>
<td>56.93 ± 3.08</td>
<td>58.14 ± 3.06</td>
<td>0.0860</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>60.32 ± 1.61 *</td>
<td>68.54 ± 2.67</td>
<td>72.02 ± 2.16</td>
<td>0.0062</td>
</tr>
<tr>
<td>dp/dt max (mmHg/s)</td>
<td>4848 ± 327 †</td>
<td>7018 ± 191 **</td>
<td>11295.57 ± 1144</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>dp/dt min (mmHg/s)</td>
<td>4295 ± 316 †</td>
<td>6182 ± 284 **</td>
<td>10198.20 ± 896</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

HW, heart weight; BW, body weight; HW:BW, ratio; TL, tibia length; maximal ATPase, actomyosin MgATPase activity (nM Pi/min/mg protein); Phospho-MBP-C, phosphorylated myosin binding protein C; phospho-TnT, phosphorylated Troponin T, LVIDd, left ventricle (LV) internal diastolic dimensions; LVIDs, LV systolic dimension; FS, fractional shortening; EF, ejection fraction; HR, heart rate; LVESP, LV end systolic pressure; LVEDP, LV end diastolic pressure; LVPD, LV developed pressure; SBP, systolic blood pressure (BP); DBP, diastolic BP; MAP, mean arterial pressure; dp/dt, maximum and minimum first derivative of LV pressure. * = p<0.05 vs. all groups, † = p<0.01 vs. all groups, § = p<0.001 vs. all groups, ‡ = p<0.05 vs. sham, ** = p<0.01 vs. sham. For myofilament analyses see Supplemental Tables I-III, Supplemental Figure I. Values analyzed by one way ANOVA, Tukey post hoc test. Mean ± SEM.
Novelty and Significance

What Is Known?

- Maintaining normal circadian rhythms is important for cardiovascular health.
- Disrupting diurnal rhythms causes and exacerbates cardiovascular disease.
- There is active diurnal rhythm disruption for patients’ post-MI in intensive care units (ICU), where lighting, noise, and frequent nocturnal assessments or medications disturb acutely ill patients.

What New Information Does This Article Contribute?

- We used a murine model of myocardial infarction to provide direct demonstration of the potential adverse consequences to long-term cardiac structure, function and ultimately outcome by even a short term disruption of diurnal rhythms (and hence disorganization of the orderly temporal sequence of repair) simulating that imposed on patients post-MI by the typical modern coronary care unit environment.
- Maintenance of normal diurnal rhythms benefits infarct healing and recovery.

Our focus on dyssynchrony studies in mice reveals that short-term rhythm disruption leads to alterations in the early inflammatory response crucial for infarct healing. Even once the animals are returned to a normal L:D environment after only 5 days post-MI, the adverse consequences of early disrupted remodeling continue. By 8 weeks post-MI the mice had significantly worse cardiac profiles by echocardiography and hemodynamics. Cardiac myofilament activity was disrupted. Histologic analyses revealed greater scar expansion and increased LV dilation as compared to animals maintained in a normal diurnal environment throughout. The circadian clock mechanism plays a role in inflammatory responses important for infarct healing. This has implications for treating patients subject to diurnal rhythm disruption, such as might occur in ICUs. These findings suggest re-evaluation and possibly some restructuring of not only contemporary intensive care but also the “diurnal health” of present-day hospital environments.
Figure 1

A

\[ \text{HW:BW (mg/g)} \]

B

\[ \text{Infarct Volume/LV Mass (mg/g)} \]

C

Sham

Normal - MI

Disrupted - MI

D

\[ \text{Infarct Volume (%LV)} \]

E

Sham

Normal - MI

Disrupted - MI

F

\[ \text{LV Diameter (Pixels X 10^2)} \]

G

\[ \text{Infarct Expansion (％)} \]
Figure 2

A) Infarct size: day 1 (%)

B) Disrupted - MI  Normal - MI

C) Respiratory Exchange Ratio

D) Respiratory Exchange Ratio

E) Corticosterone (ng/ml)

F) LVIDd (mm)
Figure 3

A. qRT-PCR myocardial mRNA expression (Fold change from Sham)

- **il-6**
  - Disrupted-MI: 33 ± 2
  - Normal-MI: 11 ± 2
  - *p < 0.05

- **mcp-1/Ccl2**
  - Disrupted-MI: 15 ± 5
  - Normal-MI: 5 ± 2
  - *p < 0.05

- **mcp-3/Ccl7**
  - Disrupted-MI: 6 ± 2
  - Normal-MI: 4 ± 2
  - *p < 0.05

B. Plasma IL-6

- Disrupted-MI: 27 pg/mL
- Normal-MI: 18 pg/mL
- *p < 0.05

C. Cardiac Myeloperoxidase

- Disrupted-MI: 37 U/mg
- Normal-MI: 16 U/mg
- *p < 0.05

D. Neutrophils

- Day 1: Disrupted-MI: 250 ± 10, Normal-MI: 200 ± 10
- Day 2: Disrupted-MI: 150 ± 10, Normal-MI: 100 ± 10
- Day 3: Disrupted-MI: 100 ± 10, Normal-MI: 50 ± 10
- *p < 0.05

E. Macrophages

- Day 3: Disrupted-MI: 300 ± 20, Normal-MI: 200 ± 20
- Day 7: Disrupted-MI: 100 ± 20, Normal-MI: 50 ± 20
- *p < 0.05
Figure 4

A. Neutrophils

- Disrupted-MI
- Normal-MI
- Sham

B. Macrophages

- Disrupted-MI
- Normal-MI
- Sham

C. Macrophages

- 2d Post-MI
- 3d Post-MI
- 7d Post-MI
Figure 5

A. CD31+ve Vessels 7d Post-MI

B. Vessel Density (vessels per 0.1mm²)

C. Day 14

D. Disrupted-MI Normal - MI

E. % Infarct Expansion

F. qRT-PCR Myocardial mRNA Expression (Fold Change vs. Sham)

G. Neutrophil (mm²)

H. Mac-3 (mm²)

I. Days Post-MI

Phase 1: Inflammatory

Phase 2: Proliferative

Phase 3: Scar Maturation
Short Term Disruption of Diurnal Rhythms Following Murine Myocardial Infarction Adversely Affects Long Term Myocardial Structure and Function
Faisal J Alibhai, Elena V Tsimakouridze, Nirmala Chinnappareddy, David C Wright, Filio Billia, Lynne O'Sullivan, W. Glen Pyle, Michael J Sole and Tami A Martino

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Supplemental Methods

Animals
Male C57Bl/6 mice (Charles Rivers, Quebec, Canada) and ClockΔ19 mice \(^1\) (ClockΔ19 are ClockΔ19/ClockΔ19 homozygous for the point mutation) were on a C57Bl/6 background (founders generously provided by Dr. Erik Herzog and Dr. Joseph Takahashi) and housed under controlled conditions in a 12-hour light (L)/12-hour dark (D) cycle with lights on at 9:30 am (Zeitgeber Time 0, ZT 0) and lights off at 9:30 pm (ZT 12). All animals were housed in a Central Animal Facility (University of Guelph). Standard rodent chow and water were provided ad libitum throughout the study. All studies were approved by the University of Guelph Institutional Animal Care and Use Committee and in accordance with the guidelines of the Canadian Council on Animal Care. ClockΔ19 mice genotyped by allele-specific PCR detecting an A-T mutation, as described\(^2\), with 5'-TGGGGTAAAAAGACCTCTTGCC-3'; 5'-GGTCAAGGGCTACAGTTCG-3'; 5'-AGCACCTTCTTTGCAATGTT-3'; 5'-TGTTGCTCAGACA-GAATAAGTA-3'. A total of 334 mice were used in this study, 96 disrupted-MI, 95 normal-MI, 61 disrupted-sham, 62 normal-sham, 7 ClockΔ19-MI and 7 wildtype (WT) littermates and 3 ClockΔ19 sham and 3 WT sham. Specific usages are outlined in the figure legends, and Supplementary Table 9, and are as follows: 1) infarct size determination at 24 hrs (n=6 per MI and n=3 per sham group); 2) Immunohistochemistry (n=4 per MI and n=3 per sham group, (for each of days 1, 2, 3, 5, 7); 3) Real time PCR for quantification of inflammatory cytokines and clock gene mRNA (n=5 per MI and n=3 per sham group (for each of time points 36h, 48h); 4) Multiplex flow cytometry for cytokines, n=5 per MI group and n=3 per sham group (for each of time points 8h, 24h, 36h, 60h, 72h, 120h, 168h), 5) Corticosterone ELISA, n=5 per MI group and n=3 per sham group (for each of time points 36h, 48h); 6) Myeloperoxidase activity, n=4 per MI group and n=3 per sham group (for each of day 1, 2, 3, 5); 7) Comprehensive lab animal monitoring system (CLAMS) for assessment of whole body metabolism, n=5 per MI group and n=3 per sham group (monitored for days 5-6); 8) Echocardiography, n=6 per MI group and n=3 per sham group (monitored from days 1-14); 9) Masson's Trichrome, infarct expansion, n=6 per MI group and n=3 per sham group (day 14); 10) Echocardiography, hemodynamics, morphometry, n=10 per MI group and n=5 per sham group (8 weeks); 11) Histopathology, n=5 per MI group and n=3 per sham group (8 weeks); 12) Myofilaments, n=5-8 per MI group and n=5-6 per sham group (8 weeks); 13) ClockΔ19, immunohistochemistry, n=7 per MI group and n=3 per sham group (day 3).

Left Anterior Descending (LAD) Coronary Artery Ligation
All surgeries were done between ZT 01 – 04. Mice (8 week old, 22-25g) were subjected to surgical ligation of the LAD coronary artery\(^3\), \(^4\). Animals were anesthetized with isoflurane, intubated, and ventilated (Harvard Apparatus model 687; St Laurent, Quebec, Canada) throughout the procedure. A 50:50 bupivacaine/lidocaine mix was administered locally prior to incision. An incision was made in the left 3rd intercostal space to expose the heart, and the pericardium gently dissected to expose the LAD. A prolene 7-0 suture (Ethicon) was passed underneath the LAD at 1mm below the edge of the left auricle and ligated. The chest and skin were then closed with silk 6-0 suture (Ethicon). Sham operations were performed the same without ligation of the LAD coronary artery. Mice were administered buprenorphine (0.1 mg/kg) upon awakening and again at 8hrs and 24hrs post-op. Following infarction mice were recovered and monitored on their regular L:D cycle until lights out, then maintained either on their normal (12-hour light (L)/12-hour dark (D)) cycle, or a disruptive (10-hour L/10-hour D) cycle. After 5 days post-MI all mice were maintained in a normal 12:12 L:D environment for up to 8 weeks,
and monitored daily. “Both groups of MI-mice these studies displayed similar survivorship (66% vs. 64% respectively) throughout the entire 8 week post-MI period.” All L:D cycles were controlled in custom made circadian cabinets.

**Evan’s Blue and TTC staining**
To measure infarct size 1 day post-MI mice were anesthetized, intubated and ventilated as described above. 1% Evan’s Blue solution was infused through the inferior vena cava to verify successful ligation of the LAD. The heart was excised, rinsed in 0.9% saline and sectioned in 1mm sections using a heart matrix (Zivic Instruments). Heart slices were incubated in 1% TTC solution for 10 minutes, transferred to 10% neutral buffered formalin overnight and photographed the next day. Infarct area and area at risk (AAR) as a percentage of left ventricle was determined using Adobe Photoshop CS4. Samples were normalized to each slice weight using the following formula; weight (total AAR) = [weight (slice 1) x % AAR (slice 1)] + [weight (slice 2) x % AAR (slice 2)] + [weight (slice n) x % AAR (slice n)]. Infarct area was calculated in a similar manner. Absolute infarct size was calculated as a ratio of weight (total infarct area) / weight (total AAR).

**Myeloperoxidase (MPO) Assay**
A separate set of hearts were collected at 1, 2, 3 and 5 days post-MI for assessment of neutrophil infiltration by MPO assay. Atria were removed, and ventricular tissue rinsed in 0.9% saline and stored at -80°C until use. MPO was isolated from the entire collected sample, in accordance with manufacturer’s directions, using the EnzChek Myeloperoxidase Activity Assay Kit (Life Technologies; Molecular Probes). Hearts were sliced into 6 pieces and homogenized in 1mL of 50 mM potassium phosphate buffer (pH 6.0) with 0.5% (w/v) hexadecyltrimethyl ammonium bromide. Homogenate was sonicated, freeze thawed 3 times and sonicated again. Samples were centrifuged at 40,000 g for 15 minutes using a Beckman Ultracentrifuge (LE-80K). Supernatant containing MPO was transferred into a fresh tube for immediate quantification. MPO values were normalized to the weight of the tissue used in each assay.

**Immunohistochemistry**
Heart sections (5um) were fixed in 10% neutral buffered formalin (NBF), paraffin embedded, and rehydrated. To assess neutrophil infiltration in the heart, antigen was retrieved using Tris-EDTA (pH9.0), sections were blocked in 3% normal goat serum (Sigma), then treated with streptavidin (Vector Labs). Sections were incubated with 1:400 rat anti-mouse anti-neutrophil (AbD, Serotec, MCA771G) overnight at 4°C. Endogenous peroxidase was quenched by 3% H2O2 for 10 min at room temperature. A 1:200 dilution of secondary antibody (goat anti-rat biotinylated antibody, Vector Labs, BA9401) was incubated with sections for 1-hour at room temperature, followed by 1-hour with 1:200 streptavidin conjugated to horse radish peroxidase (HRP) (Vector Labs, SA5004). Staining was visualized using ImmPact Nova Red (Vector Labs). To assess macrophage infiltration in the heart, antigen was retrieved using 10mM sodium citrate (pH 6.0), blocked in 3% goat serum, and treated with streptavidin. Sections were incubated with 1:50 rat anti-mouse Mac-3 (BD Biosciences, 550292) overnight at 4°C. Endogenous peroxidase was quenched, and samples were incubated with 1:200 goat anti-rat biotinylated secondary antibody (Vector Labs, BA9401) and streptavidin-HRP applied as described above. Staining was visualized with diaminobenzadine (DAB). To assess blood vessel formation in the infarct region, antigen was retrieved with Tris-EDTA (pH 9.0), sections blocked with 3% goat serum, stained with 1:400 primary antibody (rabbit anti-CD31, Abcam 28364), followed by 1:200 secondary antibody (goat anti-rabbit biotinylated, Vector Labs BA1000), then streptavidin-HRP-DAB as described above. For neutrophils and macrophages, positive stained cells were identified in a minimum of 6 fields of views at 200x magnification, using at least 3 sections per
heart. Vessels in infarcted myocardium were counted in a minimum of 4 fields of view at 400x magnification, from at least 3 sections per heart. Sections were quantified in a blinded manner.

**Blood Plasma Cytokines and Corticosterone Levels**

To investigate the effect of diurnal disruption on peripheral cytokines, mice were anesthetized with isoflurane at 8-hours, 24-hours, 48-hours, 60-hours, 72-hours, and 7 days post-surgery. Blood (1mL) was collected by left ventricular cardiac puncture into heparinized tubes, centrifuged at 1500g for 10 min, aliquoted, and stored at -80°C until use. Cytokines were quantified using the mouse flex CBA kit array (BD Biosciences) on the Accuri C6 flow cytometer (BD Biosciences) according to manufacturer’s specifications. Corticosterone was quantified from plasma collected at 24 hours, 36 hours, 48 hours and 60 hours post-surgery using the corticosterone enzyme-linked immunosorbent assay (ELISA) kit (ASSAYPRO, St Charles, MO) according to the manufacturer’s specifications. Absorbance was measured at 450nm using a Synergy HT Mutli-Mode microplate reader (BioTek). Following blood collection, animals were sacrificed by cervical dislocation, and hearts collected for immunohistochemistry.

**RNA isolation and RealTime polymerase chain reaction (RTPCR)**

Total RNA from infarcted myocardium (or sham left ventricle) was prepared using TRIZOL (Invitrogen) as described previously, and quality assessed by Nanodrop (Thermo Scientific). Amplification was performed on a VIIA7 real time PCR system (Life Technologies) using the RNA-to-Ct one step PCR kit (Life Technologies) under the following protocol: reverse transcription, 48°C for 30 min, 95°C for 10 min for 1 cycle, followed by amplification at 95°C for 15 sec, 60°C for 1 min for 40 cycles, followed by hold at room temperature. The primers used were: **matrix metalloproteinase 9 (MMP9)** forward (5'-GACATAGACGGCAGTCCAGTATC-3') and reverse (5'-GGTAGTGGGACACATAGGG-3'); **macrophage chemotactic protein (MCP1 or Ccl2)** forward (5'-GTCCTGTCACTGTTCTGG-3') and reverse (5'-TCTTGTGGATGATGAGTG-3'); **interleukin 1b (IL1b)** forward (5'-GGAGGACCATCCCAAT-3') and reverse (5'-GGTATGTGGGACACATAGGG-3'); **interleukin 6 (IL6)** forward (5'-GGATCTTTTGTTTCTTGACATAGC-3'); **tumor necrosis factor alpha (TNFa)** forward (5'-GCTGCTGTTGATGATGAGTG-3'); **chemokine (C-C motif) ligand 7 (CCL7 or MCP3)** forward (5'-TCTTGCCTCTCCTTTTCTCCAC-3') and reverse (5'-GGATCTTTTGTTTCTCGATAGC-3'); **clock** forward (5'-GAAGAGTGCGCCCTCCTCTCT-3') and reverse (5'-ACCAGTAGCAGAGCAGC-3'); **period 2 (per2)** forward (5'-TCATCTTTGGAGGGCACA-3') and reverse (5'-GCATCGAGTTGGGCGAT-3'); **nuclear factor, interleukin 3 regulated (nfil3)** forward (5'-CTTCAGACTACAGACATCCAA-3') and reverse (5'-GATGCAATTCGGCGACAT-3'); **nuclear receptor subfamily 1, group D (reverba)** forward (5'-GGGACAAGCAACATTACCA-3') and reverse (5'-GAGTAGCTCCACACACCTTAC-3'); **histone** forward (5'-GCAAAGTGCCTCTCTCTTG-3') and reverse (5'-GGCTCAGACTTGGCCCTG-3'). RTPCR was normalized to histone using the delta delta CT method.

**Non-invasive Mouse Monitoring for Whole Body Metabolism**

To investigate daily body metabolism, and daily physical activity, in normal and disrupted light cycles, we used the state-of-the-art non-invasive lab animal monitoring system (comprehensive lab animal monitoring system; CLAMS). Animals were placed in the CLAMS at 3 days post-surgery and allowed to acclimatize to the chambers for 24 hours before measurements were taken. Measurements were taken every 15 minutes over a 48 hour period (days 4 to 6). Whole body substrate utilization (respiratory exchange ratio; RER), energy...
expenditure (indirect calorimetry), food intake, and physical activity (infrared beam breaks) were measured on days 5 and day 6 post-surgery (MI and sham), and at the end of day 5 disrupted animals were transferred to a 12:12 LD without interruption of the CLAMS system. Twenty-four hour averages, as well as light vs. dark phase averages, were determined for RER (ratio of O2 intake (ml/kg/min) to CO2 production (ml/kg/min)) Energy expenditure averages (kCal/hour) were determined by taking the average of each respective phase. Total physical activity (beam breaks/15 minute bins) and total food intake (grams) were also calculated.

Echocardiography
At 1, 3, 5, 7, 14 days and 8 weeks post-MI cardiac function and morphometry were assessed in a blinded manner under light anesthesia (1.5% isoflurane) using a GE Vivid 7 Dimension ultrasound machine (GE Medical Systems) with a 14MHz linear-array transducer. All measurements were taken at the mid-papillary level. These were to determine left ventricular internal dimension at end-diastole (LVIDd), left ventricular internal dimension in systole (LVIDs), ejection fraction (EF) and fractional shortening (FS) (measured as [LVIDd-LVIDs/LVIDd] X 100) and heart rate (HR). At least 3 different images per heart were analyzed and n ≥3 hearts per group.

Hemodynamics
Hemodynamics measurements at 8 weeks post-surgery were collected in a blinded manner. Body temperature was continuously monitored and maintained. Baseline calibrations of a 1-Fr Microtip catheter (Millar) were performed as per Millar standard protocol. The carotid artery was isolated, a small incision made and the catheter advanced into the LV via the ascending aorta. Physiologic LV and aortic pressure measurements were recorded with ADInstrument PowerLab. Left ventricular end systolic pressure (LVESP), left ventricular end diastolic pressure (LVEDP), left ventricular developed pressure (LVDP) and maximum and minimum first derivative of LV pressure (dP/dtmax; dP/dtmin), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were recorded. Mean arterial blood pressure (MAP) is calculated as: DBP + (SBP-DBP)/3. Following continuous data acquisition, recorded pressures are analyzed with Lab Chart 7 (Colorado Creeks, US).

Myofilaments
Cardiac ventricular myofilaments were isolated from non-infarcted myocardium in ice-cold Standard Buffer (30 mM Imidazole (pH 7.0), 60 mM KCl, 2 mM MgCl2, 0.2 mM PMSF, 0.1 mM Leupeptin and 0.1 mM Benzamidine) as described previously. Actomyosin Mg2+ ATPase assay was measured by incubating myofilaments at 32°C for 10 min in mixtures of Activating and Relaxing buffer to create a range of free Ca2+. Activating Buffer contained 23.5 mM KCl, 20 mM Imidazole (pH 7.0), 5 mM MgCl2, 3.2 mM ATP, 2 mM EGTA, 2.2 µM CaCl2, 0.2 mM PMSF, 0.1 mM Leupeptin and 0.1 mM Benzamidine, Relaxing Buffer consisted of 26 mM KCl, 5.1 mM MgCl2, 3.2 mM ATP, 2 mM EGTA, 20 mM Imidazole (pH 7.0), 4.9 µM CaCl2. Free calcium was calculated using MaxChelator software. Reactions were quenched with ice-cold 10% trichloroacetic acid. Pi was measured by adding 0.5% FeSO4 and 0.5% ammonium molybdate in 0.5 M H2SO4) and reading the absorbance of the solution at 630nm. Sigmoidal actomyosin Mg2+ATPase activity-calcium relations were fit by a nonlinear fit procedure to a modified Hill equation: P = max ● [Ca2+]H/([Ca2+]H + EC50H) where ‘P’ is actomyosin MgATPase activity, ‘max’ is the maximum value at saturating calcium, ‘EC50’ is the calcium concentration at which 50% of maximum is reached, and ‘H’ is the slope of the relationship (Hill coefficient).
Myofilament Phosphorylation
To investigate myofilament protein phosphorylation, myofilament proteins (10 µg) were separated on 12% SDS-polyacrylamide gels, fixed in 50% methanol/10% acetic acid at room temperature overnight and stained with Pro-Q Phosphoprotein Diamond Stain (Molecular Probes, Eugene, OR, U.S.A.) according to the manufacturer’s instructions. Imaging was done using a Typhoon gel scanner (GE Healthcare, Baie Quebec). Densitometric analysis was performed on bands representing cardiac myosin-binding protein C (MyBP-C), troponin T (TnT), tropomyosin (Tm) and troponin I (TnI) using ImageJ software (NIH, Bethesda, MD, U.S.A.). Protein load was confirmed by Coomassie stain, with phosphorylation values normalized to protein load.

Troponin I phosphorylation of S23 and S24 was determined by immunoblotting. Myofilament proteins (10 µg) were separated by SDS-PAGE (12% resolving) and transferred to nitrocellulose membranes. Membranes were probed with an antibody (1:2000) for phosphorylated S23/S24 (Cell Signaling Technology) and visualized using Western Lightning (PerkinElmer Life and Analytical Sciences, Woodbridge, ON), and analysis of band density was done using ImageJ. Total protein load was measured on the same membranes using an anti-actin antibody (1:25,000; Millipore).

Morphometry and Histology
Upon sacrifice body weight (BW), heart weight (HW) and tibia length (TL) were collected from each mouse, at either 14 days or 8 weeks post-surgery. Hearts were perfused with KCl to arrest in diastole and placed into ice cold KCl, fixed in 10% neutral buffered formalin for 24 hours and placed into 70% ethanol until processing. Hearts were paraffin embedded and the entire heart sectioned (5µm) starting from apex, collecting 10 sections every 800µm. Sections were stained with Masson’s trichrome. Pictures were taken at 2.5x using Q-Capture (QImaging; Surrey, BC) and analyzed in Image J 1.46 (NIH). For infarct volume, LV mass ratio was calculated as (infarct volume as a percentage of the left ventricle/left ventricle mass)*100. Relative infarct expansion was determined by dividing the sum of the endocardial and epicardial circumference occupied by the infarct by the sum of the total LV epicardial and endocardial circumferences.

Statistical Analysis
Values are expressed as mean ± SEM. Statistical comparisons were done using unpaired students t-test, one-way or two-way analysis of variance (ANOVA) followed by Tukey test for multiple groups in Prism 6 (GraphPad Software Inc, CA). Values of p<0.05 were considered statistically significant. All values were plotted in Microsoft Excel or Sigma Plot.
Supplemental Figures

Supplemental Figure I

A

Calcium (µM)

Adenosine MyoT-Plate (formation rate)

0.01
0.1
1
10
100

MyBP-C

desmin

TnT

tropomyosin

TnI

Phosphorylation

C

Coomassie

D

E

F

Arbitrary Units (a.u.)

Phosphorylation (Arbitrary Units)

0.0
0.50
1.0
1.5
2.0
2.5
3.0

MyBP-C

Desmin

TnT

tropomyosin

TnI

Arbitrary Units (a.u.)

Sham
Normal-MI
Disrupted-MI

Ser 23/24

Actin

Sham
Normal-MI
Disrupted-MI
Supplemental Figure II

A

Day 5

Day 6

Respiratory Exchange Ratio

0.7 0.8 0.9 1.0 1.1

09:30 21:30 09:30 21:30 09:30

Clock Time (hrs)  

Normal - Sham  

Disrupted - Sham

B

Day 5

Day 6

Respiratory Exchange Ratio

0.7 0.8 0.9 1.0 1.1

09:30 21:30 09:30 21:30 09:30

Clock Time (hrs)  

Normal - Sham  

Normal - MI
Supplemental Figure III

Non-Infarcted Myocardium

CD31 +ve Vessels 7d Post-MI

Supplemental Figure IV

- **clock**
  - 36hr: 2.5 ± 0.5
  - 48hr: 3.0 ± 0.6

- **per2**
  - 36hr: 1.8 ± 0.3
  - 48hr: 2.2 ± 0.4

- **nfil3**
  - 36hr: 2.0 ± 0.2
  - 48hr: 2.5 ± 0.3

- **reverba**
  - 36hr: 1.5 ± 0.2
  - 48hr: 2.0 ± 0.3

Fold change vs. Sham

- **Disrupted- MI**
- **Normal- MI**
Supplemental Figure Legends

Supplemental Figure I. Effects of acute MI on myofilament function in mice with normal and disrupted circadian rhythms. A) Mice maintained on a normal 12:12 LD environment post-MI had an insignificant change in maximum calcium-dependent myofilament ATP consumption. Myofilaments from mice placed in a disrupted 10:10 LD environment for only 5 days post-MI showed significant (p=0.02) reductions in actomyosin MgATPase activity at free calcium concentrations greater than 10 uM versus normal-MI. B) Calcium sensitivity as measured by EC50 was insignificantly reduced following MI in both groups, as was the Hill coefficient. *p<0.05 vs. sham. n=11 (sham), n=7 (normal-MI), n=8 (disrupted-MI). For 1A and 1B, statistics were one-way ANOVA followed by Tukey’s post-hoc test. C) Cardiac myofilaments were isolated and proteins separated by SDS-PAGE. Phosphorylation of myofilament proteins was done by staining 12% acylamide gels with Pro-Q Diamond. Representative blots of each group are shown. D) Protein loading was assessed by staining gels with coomassie stain after Pro-Q Diamond staining, and normalizing to actin levels. E) Myofilaments from hearts subjected to MI and the normal 12:12 environment showed increases of 11 ± 2% in desmin, and 19 ± 5% in tropomyosin, along with an 11 ± 6% decrease in troponin I, as compared to sham. Myofilaments from disrupted-MI hearts had reductions in MyBP-C, troponin T, and troponin I phosphorylation of 25 ± 5%, 17 ± 2%, and 17 ± 5% respectively, as compared to sham hearts. There were significant differences in the phosphorylation levels of MyBP-C and troponin T between normal-MI and disrupted-MI hearts. n=10 (sham), n=5 (normal-MI), n=5 (disrupted-MI). F) Myofilament phosphorylation status can be modified by protein kinase A (PKA). Serine 23/24 of troponin I is significantly decreased in disrupted-MI myofilaments vs al other groups. For 1E and 1F, statistics were one-way ANOVA followed by Tukey’s post-hoc test. *p<0.05 vs. sham. ‡p<0.05 normal-MI vs. disrupted-MI. MyBP-C, myosin binding protein C; TnT, troponin T; TnI, troponin I.

Supplemental Figure II. Whole body substrate utilization rhythms at days 4 and 5 post-surgery. A) Respiratory exchange ratio (RER) is significantly altered by diurnal rhythm disruption in sham mice, consistent with the findings with the MI mice. That is altering the diurnal environment alters whole body metabolism in all animals studied. However, when animals are returned to a normal 24-hour environment diurnal RER rhythms are restored, as anticipated. Dotted purple line = normal-sham mice, dotted red line = disrupted-sham mice. B) Substrate utilization was not affected by surgery as the RER rhythms were the same for both normal-sham mice. Dotted purple line = normal-sham mice, solid black line = normal-MI mice. n=5 for normal-MI mice, n=3 for normal-sham mice. Values are mean ± SEM

Supplemental Figure III. CD31 staining of non-infarcted myocardium. Rich capillary vasculature of the myocardium (day 7) at 20x magnification (top panel) and 60x magnification (lower panel).

Supplemental Figure IV. RTPCR of non-infarcted myocardium. clock, per2, nfil3 and reverba mRNA (36h,48h) display altered expression in diurnal disrupted hearts, *=p<0.05, n=5 per group per time point. Values are mean ± SEM.
### Supplemental Tables

**Supplemental Table I.** Actomyosin MgATPase activity in impaired in disrupted-MI myofilaments 8 weeks post-MI compared to normal-MI

<table>
<thead>
<tr>
<th></th>
<th>Maximum Activity (nM Pi/min/mg protein)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (µM)</th>
<th>Hill Coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>213 ± 4</td>
<td>1.68 ± 0.10</td>
<td>1.61 ± 0.10</td>
</tr>
<tr>
<td>Normal - MI</td>
<td>221 ± 7</td>
<td>1.54 ± 0.10</td>
<td>1.47 ± 0.09</td>
</tr>
<tr>
<td>Disrupted - MI</td>
<td>196 ± 6*</td>
<td>1.53 ± 0.10</td>
<td>1.54 ± 0.10</td>
</tr>
</tbody>
</table>

* = p<0.05 disrupted-MI vs Sham.

**Supplemental Table II.** Myofilament protein phosphorylation in disrupted-MI myofilaments is altered compared to normal-MI

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>Normal - MI</th>
<th>% Change</th>
<th>Disrupted - MI</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>MyBP-C</td>
<td>1.76 ± 0.07</td>
<td>1.87 ± 0.07</td>
<td>↑7 ± 4</td>
<td>1.33 ± 0.07*</td>
<td>↓25 ± 5‡</td>
</tr>
<tr>
<td>Desmin</td>
<td>1.68 ± 0.04</td>
<td>1.87 ± 0.05*</td>
<td>↑11 ± 2*</td>
<td>1.70 ± 0.09</td>
<td>↑1 ± 5</td>
</tr>
<tr>
<td>Troponin T</td>
<td>2.32 ± 0.10</td>
<td>2.57 ± 0.06</td>
<td>↑11 ± 2</td>
<td>1.92 ± 0.04*</td>
<td>↓17 ± 2‡</td>
</tr>
<tr>
<td>Tropomyosin</td>
<td>1.73 ± 0.06</td>
<td>2.06 ± 0.11*</td>
<td>↑19 ± 5*</td>
<td>1.79 ± 0.04</td>
<td>↑3 ± 2</td>
</tr>
<tr>
<td>Troponin I</td>
<td>2.69 ± 0.05</td>
<td>2.41 ± 0.14*</td>
<td>↓11 ± 6*</td>
<td>2.23 ± 0.11*</td>
<td>↓17 ± 5*</td>
</tr>
</tbody>
</table>

% change = change from sham levels. * = p<0.05 vs. sham and ‡=p<0.05 vs. all other groups.

**Supplemental Table III.** Troponin I Serine 23/24 phosphorylation is decreased in disrupted-MI myofilaments compared to normal-MI

<table>
<thead>
<tr>
<th></th>
<th>Tnl Serine 23/24 Phosphorylation (Arbitrary Units; a.u.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.53±0.05</td>
</tr>
<tr>
<td>Normal-MI</td>
<td>0.39±0.04</td>
</tr>
<tr>
<td>Disrupted-MI</td>
<td>0.25±0.04‡*</td>
</tr>
</tbody>
</table>

* = p<0.05 disrupted-MI vs. sham, ‡=p<0.05 disrupted-MI vs. all groups
**Supplemental Table IV.** Diurnal disruption post-MI alters daily metabolism

### Light Phase (sleep time)

<table>
<thead>
<tr>
<th>Day</th>
<th>Disrupted MI</th>
<th>Normal MI</th>
<th>Disrupted Sham</th>
<th>Normal Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food Intake (g/hr)</td>
<td>0.11±0.02</td>
<td>0.09±0.02</td>
<td>0.19±0.03</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>Energy Expenditure (kcal)</td>
<td>0.34±0.03</td>
<td>0.36±0.03</td>
<td>0.40±0.01</td>
<td>0.39±0.01</td>
</tr>
<tr>
<td>RER</td>
<td>0.95±0.02*</td>
<td>0.88±0.01</td>
<td>0.98±0.02*</td>
<td>0.86±0.01</td>
</tr>
<tr>
<td>Activity (beam breaks/hr)</td>
<td>277.95±3.26</td>
<td>208.52±29.51</td>
<td>217.57±49.78</td>
<td>305.89±67.15</td>
</tr>
<tr>
<td><strong>Day 6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food Intake (g/hr)</td>
<td>0.08±0.01</td>
<td>0.09±0.02</td>
<td>0.11±0.02</td>
<td>0.10±0.01</td>
</tr>
<tr>
<td>Energy Expenditure (kcal)</td>
<td>0.35±0.03</td>
<td>0.37±0.03</td>
<td>0.41±0.02</td>
<td>0.41±0.01</td>
</tr>
<tr>
<td>RER</td>
<td>0.90±0.02</td>
<td>0.88±0.01</td>
<td>0.92±0.02</td>
<td>0.86±0.01</td>
</tr>
<tr>
<td>Activity (beam breaks/hr)</td>
<td>237.65±25.53</td>
<td>214.85±42.39</td>
<td>277.69±44.27</td>
<td>278.17±34.48</td>
</tr>
</tbody>
</table>

RER; Respiratory Exchange Ratio, *=p<0.05 disrupted-MI vs. normal-MI. ‡=p<0.05 disrupted-Sham vs. normal-Sham

### Dark Phase (wake time)

<table>
<thead>
<tr>
<th>Day</th>
<th>Disrupted MI</th>
<th>Normal MI</th>
<th>Disrupted Sham</th>
<th>Normal Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Day 5</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food Intake (g/hr)</td>
<td>0.25±0.02</td>
<td>0.20±0.03</td>
<td>0.31±0.02</td>
<td>0.24±0.01</td>
</tr>
<tr>
<td>Energy Expenditure (kcal)</td>
<td>0.39±0.03</td>
<td>0.42±0.03</td>
<td>0.46±0.02</td>
<td>0.49±0.02</td>
</tr>
<tr>
<td>RER</td>
<td>0.99±0.01*</td>
<td>0.93±0.02</td>
<td>1.03±0.02*</td>
<td>0.97±0.01</td>
</tr>
<tr>
<td>Activity (beam breaks/hr)</td>
<td>1069.83±95.94</td>
<td>890.21±58.38</td>
<td>1150.60±138.74</td>
<td>1665.58±183.81</td>
</tr>
<tr>
<td><strong>Day 6</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food Intake (g/hr)</td>
<td>0.18±0.02</td>
<td>0.19±0.03</td>
<td>0.24±0.01</td>
<td>0.25±0.01</td>
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<tr>
<td>Energy Expenditure (kcal)</td>
<td>0.42±0.03</td>
<td>0.42±0.03</td>
<td>0.48±0.02</td>
<td>0.52±0.01</td>
</tr>
<tr>
<td>RER</td>
<td>0.92±0.01</td>
<td>0.95±0.02</td>
<td>0.95±0.01</td>
<td>0.97±0.01</td>
</tr>
<tr>
<td>Activity (beam breaks/hr)</td>
<td>1082.83±187.64</td>
<td>1015.77±180.85</td>
<td>995.69±176.99</td>
<td>1996.14±261.21</td>
</tr>
</tbody>
</table>

RER; Respiratory Exchange Ratio, *=p<0.05 disrupted-MI vs. normal-MI. ‡=p<0.05 disrupted-sham vs. normal-sham
**Supplemental Table V.** Echocardiography shows exacerbated LV remodeling in disrupted mice

<table>
<thead>
<tr>
<th>Day</th>
<th>Disrupted-MI*</th>
<th>Normal-MI*</th>
<th>Disrupted-Sham</th>
<th>Normal– Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LVIDd (mm)</strong></td>
<td>4.38±0.05</td>
<td>4.32±0.08</td>
<td>3.86±0.09</td>
<td>3.97±0.04</td>
</tr>
<tr>
<td><strong>LVIDs (mm)</strong></td>
<td>3.39±0.08</td>
<td>3.22±0.11</td>
<td>2.19±0.08</td>
<td>2.26±0.03</td>
</tr>
<tr>
<td><strong>EF (%)</strong></td>
<td>55.52±2.00</td>
<td>58.20±2.06</td>
<td>80.59±1.80</td>
<td>80.04±0.45</td>
</tr>
<tr>
<td><strong>FS (%)</strong></td>
<td>24.91±1.12</td>
<td>25.67±1.46</td>
<td>43.90±1.80</td>
<td>42.90±0.45</td>
</tr>
</tbody>
</table>

**Day 3**

| **LVIDd (mm)** | 4.55±0.06 | 4.63±0.09 | 3.95±0.06 | 4.07±0.02 |
| **LVIDs (mm)** | 3.31±0.08 | 3.33±0.10 | 2.35±0.02 | 2.33±0.01 |
| **EF (%)** | 59.64±1.89 | 60.50±2.28 | 77.53±0.86 | 79.90±0.34 |
| **FS (%)** | 27.43±1.19 | 28.21±1.53 | 40.49±0.75 | 42.76±0.34 |

**Day 5**

| **LVIDd (mm)** | 5.04±0.11 | 4.89±0.15 | 4.00±0.06 | 4.05±0.04 |
| **LVIDs (mm)** | 3.80±0.19 | 3.53±0.20 | 2.24±0.10 | 2.33±0.07 |
| **EF (%)** | 54.86±3.59 | 57.22±4.24 | 81.13±1.55 | 79.72±1.33 |
| **FS (%)** | 24.87±2.14 | 28.53±2.21 | 43.97±1.57 | 42.57±1.34 |

**Day 7**

| **LVIDd (mm)** | 5.35±0.12** | 4.94±0.10 | 4.05±0.01 | 4.06±0.04 |
| **LVIDs (mm)** | 4.13±0.16 | 3.73±0.15 | 2.39±0.06 | 2.35±0.08 |
| **EF (%)** | 51.92±2.41 | 54.99±2.83 | 78.13±1.56 | 78.97±1.65 |
| **FS (%)** | 23.01±1.39 | 24.69±1.47 | 41.08±1.47 | 42.03±1.76 |

**Day 14**

| **LVIDd (mm)** | 5.65±0.15** | 5.22±0.10 | 4.04±0.07 | 3.96±0.06 |
| **LVIDs (mm)** | 4.37±0.19 | 3.92±0.12 | 2.32±0.07 | 2.26±0.06 |
| **EF (%)** | 52.08±3.42 | 54.76±2.78 | 79.67±1.01 | 80.00±0.87 |
| **FS (%)** | 23.28±1.96 | 25.58±1.07 | 42.47±0.95 | 42.87±0.85 |

- **LVIDd/LVIDs**, left ventricular internal dimensions diastole/ systole; **EF**, ejection fraction; **FS**, fractional shortening; **AvHR**, average heart rate; **BW**, body weight; **HW**, heart weight; **HW/BW**, heart weight:body weight ratio, **HW/TL**; heart weight: tibia length ratio. The same animals were followed for 14 days post-surgery and sacrificed for morphometry (BW, HW and TL) at day 14.
- *=p<0.05 MI vs. Sham, all parameters except HR and BW. **=p<0.05 disrupted-MI vs. all other groups by one way ANOVA followed by a Tukey post-hoc test. Values are mean ± SEM.
**Supplemental Table VI.** Disruption post-MI alters inflammatory cytokine mRNA expression in the infarcted myocardium

<table>
<thead>
<tr>
<th>Gene</th>
<th>Disrupted-MI</th>
<th>Normal-MI</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>25.80±4.73*</td>
<td>8.11±1.29</td>
</tr>
<tr>
<td>MCP-1</td>
<td>9.63±1.03*</td>
<td>5.84±1.13</td>
</tr>
<tr>
<td>CCL7</td>
<td>3.42±0.48*</td>
<td>1.83±0.47</td>
</tr>
<tr>
<td>IL-1b</td>
<td>13.78±0.83</td>
<td>10.15±1.91</td>
</tr>
<tr>
<td>TNFa</td>
<td>12.65±0.96</td>
<td>11.48±1.30</td>
</tr>
<tr>
<td>MMP9</td>
<td>23.29±2.65</td>
<td>20.94±2.03</td>
</tr>
</tbody>
</table>

*=p<0.05 disrupted-MI vs. normal-MI. All values were analyzed using the ΔΔCT method with normalization to histone and expressed as a fold change vs. sham groups.

**Supplemental Table VII.** Plasma IL-6 levels are elevated by diurnal rhythm disruption post-MI.

<table>
<thead>
<tr>
<th>Time Post-Surgery (hours)</th>
<th>Disrupted-MI (pg/mL)</th>
<th>Normal-MI (pg/mL)</th>
<th>Disrupted-Sham (pg/mL)</th>
<th>Normal-Sham (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>35.25±4.96*</td>
<td>43.09±4.47</td>
<td>3.613±1.82</td>
<td>1.73±0.42</td>
</tr>
<tr>
<td>24</td>
<td>30.03±8.44*</td>
<td>23.26±7.95</td>
<td>11.30±3.58</td>
<td>7.95±1.94</td>
</tr>
<tr>
<td>36</td>
<td>17.90±4.91*</td>
<td>4.92±1.19</td>
<td>3.79±1.68</td>
<td>2.25±0.38</td>
</tr>
<tr>
<td>48-60</td>
<td>38.69±10.43*</td>
<td>10.17±2.35</td>
<td>0.51±0.34</td>
<td>2.32±0.5</td>
</tr>
<tr>
<td>72</td>
<td>12.16±2.23</td>
<td>12.46±0.85</td>
<td>Below Detection</td>
<td>Below Detection</td>
</tr>
<tr>
<td>120</td>
<td>3.03±1.96</td>
<td>Below Detection</td>
<td>Below Detection</td>
<td>Below Detection</td>
</tr>
<tr>
<td>168</td>
<td>2.54±1.29</td>
<td>Below Detection</td>
<td>Below Detection</td>
<td>Below Detection</td>
</tr>
</tbody>
</table>

*=p<0.05 disrupted-MI vs. normal-MI, and ‡=p<0.05 disrupted-MI vs. sham groups, by two way ANOVA followed by a Tukey post hoc. n=5 per MI group and n=3 per sham group for all time points. Values are mean ± SEM.

**Supplemental Table VIII.** Cardiac myeloperoxidase is altered in disrupted-MI hearts

<table>
<thead>
<tr>
<th>Days Post-Surgery</th>
<th>Disrupted-MI† (ng/mL/100mg)</th>
<th>Normal-MI† (ng/mL/100mg)</th>
<th>Disrupted-Sham (ng/mL/100mg)</th>
<th>Normal-Sham (ng/mL/100mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>39.07±1.84</td>
<td>32.30±4.25</td>
<td>12.35±0.64</td>
<td>12.46±0.35</td>
</tr>
<tr>
<td>2</td>
<td>50.79±5.19*</td>
<td>85.89±10.76</td>
<td>18.24±1.03</td>
<td>16.85±1.24</td>
</tr>
<tr>
<td>3</td>
<td>44.39±3.23</td>
<td>50.28±4.26</td>
<td>12.62±2.34</td>
<td>11.12±1.14</td>
</tr>
<tr>
<td>5</td>
<td>55.60±2.39*</td>
<td>26.56±1.12</td>
<td>9.85±1.05</td>
<td>8.1±0.43</td>
</tr>
</tbody>
</table>

*=p<0.05 disrupted-MI vs. normal-MI, and †=p<0.05 vs. sham groups by two way ANOVA followed by a Tukey post hoc (n=4 per group) Values are mean ± SEM. Additionally, two way ANOVA reveals that the light dark cycle significantly (p<0.05) affects MPO activity post-MI. Furthermore, there is a significant (p<.01) interaction between the L:D cycle and MPO activity over 5 day post-MI; that a disrupted diurnal cycle significantly affects temporal MPO activity over the first 5 days post-MI.
### Supplemental Table IX. All endpoints assessed with specific times and n values

<table>
<thead>
<tr>
<th>Endpoint</th>
<th>Disrupted-MI (n)</th>
<th>Normal-MI (n)</th>
<th>Disrupted-Sh (n)</th>
<th>Normal-Sh (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infarct Size Determination (24hr)</td>
<td>6</td>
<td>6</td>
<td>3</td>
<td>3</td>
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<tr>
<td>Immunohistochemistry (IHC) (Day 1)</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>IHC (Day 2)</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>IHC (Day 3)</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>IHC (Day 5)</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>IHC (Day 7)</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Real Time PCR (RTPCR) (36hr)</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
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<tr>
<td>RTPCR (48hr)</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Multiplex flow cytometry for cytokines (8hr)</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Cytokines, corticosterone ELISA (24hr)*</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Cytokines, corticosterone ELISA (36hr)</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Corticosterone ELISA (48hr)*</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Cytokines (60hr)</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Cytokines (72hr)*</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Cytokines (120hr)*</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Cytokines (168hr)*</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Myeloperoxidase (MPO) activity (Day 1)</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
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<tr>
<td>MPO activity (Day 2)</td>
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<td>4</td>
<td>3</td>
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<tr>
<td>MPO activity (Day 3)</td>
<td>4</td>
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<td>MPO activity (Day 5)</td>
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<td>4</td>
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<tr>
<td>CLAMS (Day 5-6)</td>
<td>5</td>
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<td>3</td>
<td>3</td>
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<tr>
<td>Echocardiography (Day 1-14)</td>
<td>6</td>
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<td>3</td>
<td>3</td>
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<tr>
<td>Masson’s Trichrome, Infarct Expansion (Day 14)*</td>
<td>6</td>
<td>6</td>
<td>3</td>
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</tr>
<tr>
<td>Morphometry (8w)*</td>
<td>10</td>
<td>10</td>
<td>5</td>
<td>5</td>
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<tr>
<td>Histopathology (8w)*</td>
<td>5</td>
<td>5</td>
<td>3</td>
<td>3</td>
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<tr>
<td>Myofilament ATPase (8w)</td>
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<td>7</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>Myofilament Phosphorylation (8w)*</td>
<td>5</td>
<td>5</td>
<td>5</td>
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</tr>
<tr>
<td>Total Used per group (excluding * = multiple tissues collected from same animal)</td>
<td>96</td>
<td>95</td>
<td>61</td>
<td>62</td>
</tr>
</tbody>
</table>

### Additional Table

<table>
<thead>
<tr>
<th></th>
<th>ClockΔ19-MI</th>
<th>WT-MI</th>
<th>ClockΔ19-Sh</th>
<th>WT-Sh</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC - Neutrophil (Day 3)</td>
<td>7</td>
<td>7</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>IHC - Macrophage (Day 3)*</td>
<td>7</td>
<td>7</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Total Used per group (excluding * = multiple tissues collected from same animal)</td>
<td>7</td>
<td>7</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

* = multiple tissues collected from same animals. Overall total number of animals used 96+95+61+62+7+7+3+3= 334
Supplemental References


