Post-infarct sleep disruption and its relation to cardiac remodeling in a rat model of myocardial infarction

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ABSTRACT
Sleep disruption after myocardial infarction (MI) by affecting ubiquitin–proteasome system (UPS) is thought to contribute to myocardial remodeling and progressive worsening of cardiac function. The aim of current study was to test the hypothesis about the increased risk of developing heart failure due to experience of sleep restriction (SR) after MI. Male Wistar rats (n = 40) were randomly assigned to four experimental groups: (1) Sham, (2) MI, (3) MI and SR (MI + SR) (4) Sham and SR (Sham + SR). MI was induced by permanent ligation of left anterior descending coronary artery. Twenty-four hours after surgery, animals were subjected to chronic SR paradigm. Blood sampling was performed at days 1, 8 and 21 after MI for determination of serum levels of creatine kinase-MB (CK-MB), corticosterone, malondialdehyde (MDA) and nitric oxide (NO). Finally, at 21 days after MI, echocardiographic parameters and expression of MuRF1, MaFBx, A2O, eNOS, iNOS and NF-kB in the heart were evaluated. We used H&E staining to detect myocardial hypertrophy. We found out that post infarct SR increased corticosterone levels. Our results highlighted deteriorating effects of post-MI SR on NO production, oxidative stress, and echocardiographic indexes (p < 0.05). Moreover, its detrimental effects on myocardial damage were confirmed by overexpression of MuRF1, MaFBx, iNOS and NF-kB (p < 0.001) in left ventricle and downregulation of A20 and eNOS (p < 0.05). Furthermore, histological examination revealed that experience of SR after MI increased myocardial diameter as compared to Sham subjects (p < 0.05). Our data suggest that SR after MI leads to an enlargement of the heart within 21 days, marked by an increase in oxidative stress and NO production as well as an imbalance in UPS that ultimately results in cardiac dysfunction and heart failure.

1. Introduction
Alterations in cardiac function play a key role in the pathogenesis and progression of myocardial infarction (MI) and heart failure (HF) (Kelly et al., 2008). Histopathological and structural changes in the left ventricular (LV) myocardium lead to LV remodeling (Lin et al., 2016) which is a compensatory and adaptive response of cardiac tissue to restructure and reshape itself and blunt LV pump dysfunction (de Waard et al., 2010). However, progressive and decompensated response of LV remodeling (i.e. prolonged pathologic cardiac hypertrophy, LV dilatation and systolic/diastolic dysfunction) seems to occur due to gradual and maladaptive changes of cellular elements of the myocardium and cause increased risk of HF and mortality (Tsutsui et al., 2011). Although this process of complete intracellular contractile apparatus reorganization leads to deterioration of ventricular function, the underlying mechanisms of this phenomenon are not fully understood (Azevedo et al., 2015). The direct or indirect involvement of the ubiquitin–proteasome system (UPS) to the cardiac remodeling after MI linked with the turnover of skeletal muscle proteins either in normal or pathologic processes has been established in different studies (Chondrogianni & Gonos, 2011). Coordination of three main enzymes of this system including ubiquitin activating enzyme (E1), transfer to an ubiquitin conjugating enzyme
(E2), and subsequent linkage to the lysine residue of proteins destined for degradation (E3) ligases causes ubiquitination of the specific protein substrates which in turn will be recognized by proteasomes and be degraded (Razeghi et al., 2006). Deubiquitylating enzymes (DUBs) remove ubiquitin residues from these substrates and affect their interactions with the proteasome. An imbalance between the expression and activity of E3 ubiquitin ligases and DUBs leads to impaired UPS which results in activation of the nuclear factor of B (NF-κB) related to increased ubiquitination and proteasome-dependent degradation of the multimeric inhibitor of κB (IκB) for development of cardiac hypertrophy (Patterson et al., 2007). Recent literature has also reported the role of nitric oxide (NO) in many processes contributing to LV remodeling and it may reduce excessive myocardial oxidative (NO) in many processes contributing to LV remodeling and it may reduce excessive myocardial oxidative stress process, myocardial hypertrophy and destroy myocardial cells to predict the later occurrence of HF. Therefore, given the critical role of UPS, NO and markers of oxidative stress in the progression of HF current study was aimed to test the hypothesis about the increased risk of developing HF after post-MI SR in a rat model of MI and accordingly to consider these factors for our future studies.

2. Experimental procedures

2.1. Study design

Forty naive, adult male Wistar rats (weighting 250–300 g) were the experimental subjects with free access to water and a standard rat diet. Ambient temperature was maintained between 22 and 24 °C, and the vivarium was maintained under a 12:12 h light/dark cycle. Before the study, the rats were assimilated into the air-conditioned colony room for at least 7 days. The experimental protocol was approved by Institutional Animal Care and Use Committee (IACUC) at the Tehran University of Medical Sciences (Iran) and complied with the Guide for the Care of Use of Laboratory Animals’ published by the US National Institute of Health (NIH Publication no. 85-23, revised 2011).

Rats were anesthetized with ketamine (50 mg/kg, intraperitoneally [i.p.]) and xylazine (10 mg/kg, i.p.), and then, MI was induced by permanently ligating the left anterior descending (LAD) approximately 3 mm from its origin using a 6-0 polypropylene suture. Sham surgery was performed in the same way as described for MI with exception of coronary artery ligation (Azizi et al., 2013). All animals received postoperative acetaminophen and cefazolin (200 and 40 mg/kg, i.p., respectively) for 3 days. After surgery, all animals assigned to four experimental groups comprising each of 10 rats: (1) Sham, (2) MI, (3) MI and SR (MI + SR) and (4) Sham and SR (Sham + SR).

Twenty-four hours after surgery and total recovery from anesthesia, animals from MI + SR and Sham + SR groups were subjected to chronic SR paradigm using Multiple Small Platform (SR-MSP) for six consecutive days: the rats were placed in this platform for 20 h every day and just allowed to sleep in their individual home cages for 4 h (Koban, 2009). Effectiveness of SR-MSP method for significantly abolishing REM sleep is validated in electroencephalography studies, although it can also disrupt slow-wave sleep (Ma et al., 2014). Before surgery, 8 and 21 days after surgery body weights were recorded. Three weeks after surgery, animals were submitted to an echocardiographic study and euthanasia for isolation of the heart. The euthanasia was induced with sodium thiopental 85 mg/kg. Five isolated samples from remote
region of myocardium in each group were clipped on ice and weighted and then directly were snap-frozen in liquid nitrogen and stored at −70 °C until ready for further use and analysis. The remaining five isolated samples in each group were also weighted and then fixed in 10% formalin for histological and immunohistochemistry assessment. The heart weight (g) was divided by the body weight (g) of 21st days after surgery and multiplying by 100; and finally, heart weight to body weight ratio was obtained.

2.2. Blood sampling

Blood samples were collected at days of 1, 8 and 21 after surgery to measure CK-MB (marker of myocardial damage), MDA (marker of oxidative stress), corticosterone (marker of stress response) and NO concentrations in the serum.

2.3. Echocardiographic assessments

Two-dimensional and M-mode images were obtained using a 10-MHz transducer connected to an ultrasonic echocardiographic system (Clear Canvas by Synaptive Medical Toronto, Canada). Interventricular septal end diastole and end systole (IVSd and IVSs), maximum minor axis of the left ventricle at end-diastole (LVDd) and end-systole (LVDs), left-ventricular post wall thickness in systole and diastole (LVPWs and LVPWd), fractional shortening (FS) and ejection fraction (EF) were obtained from the M-mode view by an independent observer experienced in rodent imaging and blinded to the experimental groups. For each measurement, three consecutive cardiac cycles were measured and averaged.

2.4. CK-MB Measurement

Serum levels of CK-MB at day 1 after surgery were measured calorimetrically with specific CK-MB kits (Pars Azmun Co, Karaj, Iran), using an auto analyzer (Roche Hitachi Modular DP Systems, Germany). All procedures were performed according to the manufacturer’s instructions. The recorded values are presented in U/l (Azizi et al., 2013).

2.5. Assessment of oxidative stress and NO

To evaluate the oxidative damage, MDA levels were assessed by thiobarbituric acid reactive substances assay (Azizi et al., 2013). Moreover, NO concentration was measured using Griess method (Mard et al., 2014).

2.6. Corticosterone assay

A commercial serum corticosterone radioimmunoassay kit (ZB-CRT-R9648; ZellBio GmbH, Germany) was used to evaluate serum concentration of corticosterone in all rats at 1 and 8 days after surgery according to the manufacturer’s instructions.

2.7. Real-time quantitative reverse transcription-polymerase chain reaction

Total RNA, frozen in liquid nitrogen, was extracted from the non-infarcted zone of LV using a GeneAllR Ribox™ Total RNA extraction kit (301-001; GeneAll Biotechnology, Seoul, Republic of Korea) according to the manufacturer’s instructions. Purity of RNA was determined with a 260/280-nm absorbance ratio data using a NanoDrop UV/vis spectrophotometer (Thermo Scientific, Waltham, MA, USA). cDNA was synthesized by reverse transcription using PrimerScript™ Reagent Kit (RR037A; TakaRa, Otsu, Japan). Quantitative real-time polymerase chain reaction (PCR) on a Corbett Rotor-Gene 6000 instrument (QIAGEN Rotor-Gene Q) was performed using the following rat-specific sense and antisense primers: muscle RING finger 1 (MuRF1), 5′-ATCAAGAAGAGGGCTGTCC-3′ (forward) and 5′-AGACACACTTTCCCTATGGTG-3′ (reverse); muscle atrophy F-box (MaFBx), 5′-AGCTTTCAACAGACTGGACCTTCT-3′ (forward) and 5′-TGTGAGCTGTGACCTTTGCTATCA-3′ (reverse); Tumor Necrosis Factor Alpha Induced Protein 3 (A20), 5′-CTCGAGCGGTGGACACAG-3′ (forward) and 5′-GCACGGATGTGTCCTGAAT-3′ (reverse); eNOS, 5′-CTGGAGCTGTGACCTTTGCTATCA-3′ (forward) and 5′-CTGAGCTGTGACCTTTGCTATCA-3′ (reverse); HPRT, 5′-CTCATGGACTGATTATGGACAGGAC-3′.
(forward) and 5′-GCAGGTAGCAAAGAATTAT AGCC-3′ (reverse). Specificity of product for each separate sample was checked with the melting curve analysis and quantification was achieved with the comparative threshold cycle method.

2.8. Immunohistochemistry staining

The hearts were fixed in 10% paraformaldehyde for 4 h, dehydrated in 30% sucrose (4 °C overnight), embedded in in paraffin and cut into 5 μm sections. For fluorescent immunohistochemistry primary antibodies (Santa Cruz Biotechnology, USA) including p-NF-κB rabbit polyclonal antibody and goat monoclonal antibodies to iNOS were used. The slides were visualized using a fluorescence microscope (Olympus BX51). Images were captured using a digital camera (Olympus DP72) and quantified using the NIH ImageJ analysis program (NIH, Bethesda, MD, USA) (Ramroodi et al., 2015).

2.9. Histological analysis

Transverse serial sections (5.0 μm thick), at the mid-papillary muscle level, were also cut from the paraffin-embedded LV slices and stained with hematoxylin and eosin (H&E) to assess myocardial hypertrophy. Using a binocular microscope (Optiphot, Nikon, Melville, NY) with a camera and image processor (DKC5000, Sony, New York, NY), images were captured and quantified using the NIH ImageJ analysis program (NIH, Bethesda, MD, USA).

2.10. Statistical analysis

Statistical analyses were performed using the SPSS software version 20 (SPSS Inc., Chicago, IL, USA). Analyses included one-way or two-way ANOVA test as appropriate and followed by the post hoc Tukey’s test for multiple comparisons. Additionally, a one-way ANOVA for repeated measures was used to test the difference between days of 1, 8 and 21 after surgery in each group. A significance level of \( p < 0.05 \) was used in all cases. Data are presented as mean ± SEM.

3. Results

3.1. Mortality and survival

There was no mortality in Sham and Sham + SR groups. Totally, four animals died during study, one animal in MI group at first 24 h after MI induction (1/10) and three animals in MI + SR group (3/10) following experience of SR.

3.2. Body and heart weights

All experimental animals were similar in body weight before surgery; however, SR paradigm caused an obvious weight loss \(( p < 0.001 \) of MI + SR and Sham + SR animals from their initial body weight (Figure 1), and the weight of these subjects was clearly lower than MI group at 8 days post-surgery \(( p < 0.01 \). After SR protocol, these animals initiated to gain weight and their body weight reached to amounts near the Sham and MI groups at 21 days post-surgery, i.e. there was no statistical difference between all experimental groups. There was a rising trend of body weight gain from day 1 to day 21 after surgery for Sham and MI animals \(( p < 0.05 \). Table 1 shows that experience of SR increased the heart weight and heart/body weight ratio 21 days after surgery in MI + SR rats compared with all experimental groups \(( p < 0.05 \). However, in sham animals which experienced post-surgery SR, the heart weight and heart/body weight ratio was considerably lower than Sham and MI groups \(( p < 0.05 \).

3.3. Echocardiographic findings

The main echocardiographic parameters measured 21 days after surgery are summarized in Table 2. Except for LVPWs and LVPWd, MI induction caused significant decrease of IVSd, IVSs, EF and FS as well as significant increase of LVDd and LVDs in both MI and MI + SR groups \(( p < 0.001 \) for all comparisons) compared with the Sham group. Furthermore, most of these parameters were similar in both MI and MI + SR subjects; however, EF and FS significantly decreased in MI + SR group rather than MI subjects \(( p = 0.002 \) and \( p = 0.044 \) for EF and FS, respectively).
3.4. Effect of MI on CK-MB levels

Induction of MI significantly increased serum levels of CK-MB ($p < 0.001$) as compared to sham operated animals (Figure 2).

3.5. Evaluation of oxidative stress and NO

As shown in Figure 3, induction of MI significantly increased MDA levels at day 1 post-surgery ($p < 0.001$) in comparison with Sham group; however, it decreased significantly 8 days after surgery in MI group ($p < 0.001$). Experience of SR after sham surgery or after MI significantly increased MDA levels 8 days post-surgery ($p < 0.001$) comparing Sham group; and this elevation was more obvious in MI + SR subjects compared with SR group ($p = 0.017$). Moreover, serum concentrations of MDA at 8 days after surgery in Sham + SR and MI + SR group were considerably higher than MI group ($p < 0.001$). Twenty-one days after surgery, MDA levels decreased significantly in

### Table 1. Heart weight and heart/body weight ratio 21 days after surgery ($n = 10$).

<table>
<thead>
<tr>
<th>Groups</th>
<th>Heart weight (g)</th>
<th>Heart/Body weight ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>0.897 ± 0.0187</td>
<td>0.00318 ± 0.000062</td>
</tr>
<tr>
<td>MI</td>
<td>0.875 ± 0.109</td>
<td>0.00314 ± 0.000066</td>
</tr>
<tr>
<td>Sham + SR</td>
<td>0.789 ± 0.008$^a$$^b$</td>
<td>0.0029 ± 0.000039$^a$$^b$</td>
</tr>
<tr>
<td>MI + SR</td>
<td>0.959 ± 0.015$^{a-c}$</td>
<td>0.00359 ± 0.000054$^{a-c}$</td>
</tr>
</tbody>
</table>

MI: Myocardial infarction; SR: sleep restriction.

$^a$ $p < 0.05$ compared to sham group.

$^b$ $p < 0.05$ compared to MI group.

$^c$ $p < 0.05$ compared to sham + SR group.

### Table 2. Echocardiographic parameters 21 days after surgery ($n = 10$).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Sham</th>
<th>MI</th>
<th>Sham + SR</th>
<th>MI + SR</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVSs (cm)</td>
<td>0.228 ± 0.01</td>
<td>0.109 ± 0.016$^a$</td>
<td>0.221 ± 0.011$^b$</td>
<td>0.086 ± 0.022$^{a-c}$</td>
</tr>
<tr>
<td>IVSd (cm)</td>
<td>0.162 ± 0.006</td>
<td>0.084 ± 0.012$^a$</td>
<td>0.154 ± 0.013$^b$</td>
<td>0.067 ± 0.014$^{a-c}$</td>
</tr>
<tr>
<td>LVDs (cm)</td>
<td>0.379 ± 0.016</td>
<td>0.657 ± 0.057$^a$</td>
<td>0.344 ± 0.03$^b$</td>
<td>0.718 ± 0.047$^{a-c}$</td>
</tr>
<tr>
<td>LVDd (cm)</td>
<td>0.671 ± 0.011</td>
<td>0.87 ± 0.04$^a$</td>
<td>0.608 ± 0.036$^b$</td>
<td>0.869 ± 0.046$^{a-c}$</td>
</tr>
<tr>
<td>LVPWs (cm)</td>
<td>0.242 ± 0.011</td>
<td>0.255 ± 0.008</td>
<td>0.242 ± 0.012</td>
<td>0.283 ± 0.01</td>
</tr>
<tr>
<td>LVPWd (cm)</td>
<td>0.157 ± 0.008</td>
<td>0.18 ± 0.015</td>
<td>0.185 ± 0.013</td>
<td>0.172 ± 0.016</td>
</tr>
<tr>
<td>EF (%)</td>
<td>79.87 ± 1.96</td>
<td>43.42 ± 2.37$^a$</td>
<td>77.8 ± 3.74$^b$</td>
<td>30.14 ± 1.37$^{a-c}$</td>
</tr>
<tr>
<td>FS (%)</td>
<td>43.5 ± 1.45</td>
<td>22.25 ± 0.77$^a$</td>
<td>41.6 ± 3.52$^b$</td>
<td>16.14 ± 0.67$^{a-c}$</td>
</tr>
</tbody>
</table>

MI: Myocardial infarction; SR: sleep restriction; IVSd and IVSs: interventricular septal end diastole and end systole, LVDd and LVDs: maximum minor axis of the left ventricle at end-diastole and end-systole; LVPWs and LVPWd: left-ventricular post wall thickness in systole and diastole; FS: fractional shortening (FS); EF: ejection fraction.

$^a$ $p < 0.05$ compared to sham group.

$^b$ $p < 0.05$ compared to MI group.

$^c$ $p < 0.05$ compared to sham + SR group.
Sham + SR group ($p < 0.001$); however, it was higher in MI + SR animals as compared with other experimental groups ($p < 0.05$). In addition, MDA levels at day 21st post-surgery in SR experienced animals ($p < 0.001$) were significantly higher as compared to MI group.

Figure 3 also shows that 1 day after surgery, NO levels were greater in MI subjects ($p < 0.001$) than Sham rats. Post-surgery SR for 6 days significantly increased serum levels of NO at 8th day after surgery in MI + SR ($p = 0.026$) and Sham + SR ($p < 0.001$) as compared to the 1st day after surgery, and these amounts were considerably higher comparing MI group ($p < 0.001$). However, there was considerable decline of NO serum concentration 8 days after LAD ligation in MI group ($p = 0.003$) comparing day 1 post-MI. Serum levels of NO in MI + SR ($p = 0.003$) and Sham + SR ($p = 0.017$) decreased 21 days after surgery as compared to 8th post-surgery and reached to amounts as similar as Sham and MI groups; i.e. there was no statistical difference between SR experienced subjects (Sham + SR and MI + SR groups) and MI group at 21 days after surgery.

### 3.6. Evaluation of corticosterone levels

Evaluation of corticosterone concentration in serum of experimental subjects (Figure 4) revealed that experience of SR after sham surgery or after MI significantly increased corticosterone levels 8 days after surgery in both Sham + SR and MI + SR subjects ($p < 0.001$) as compared to day 1 post-surgery and this increase was more obvious in MI + SR group in comparison with Sham + SR group ($p = 0.022$).

### 3.7. mRNA expression of MuRF1, MaFBx, A20, eNOS and iNOS in the heart

Figure 5 reveals all changes in mRNA expression of MuRF1, MaFBx, A20, eNOS and iNOS genes in the
heart at 21 days after surgery. A significant increase in A20 \((p = 0.002)\) and MuRF1 \((p = 0.004)\), MaFBx \((p = 0.007)\) and eNOS \((p = 0.001)\) gene expression was observed in MI group compared to Sham group. However, iNOS expression in MI group was not different to Sham group \((p = 0.351)\). Although MuRF1 \((p = 0.004)\), MaFBx \((p = 0.031)\) and iNOS \((p < 0.001)\) genes upregulated in MI + SR subjects as compared to Sham group, we observed a significant decrease in A20 \((p < 0.001)\) and eNOS \((p = 0.001)\) gene expression in these subjects comparing Sham rats. Statistical analysis also showed a significant decrease in eNOS as well as increase in A20 \((p < 0.001)\) and iNOS \((p < 0.001)\) expression in Sham rats which experienced SR after surgery compared to Sham group. Although MuRF1 \((p = 0.052)\) and MaFBx \((p = 0.133)\) expression in Sham + SR group was not different to Sham group, these two genes were considerably expressed in MI + SR subjects as compared to MI group \((p < 0.001)\). Comparing MI and MI + SR groups reveled that A20 and eNOS \((p < 0.001)\) significantly downregulated in MI + SR rats.
3.8. Immunohistochemistry study

Compared with the Sham group, immunohistochemistry results (Figure 6) revealed that p-NF-κB and iNOS expression ($p < 0.001$) in MI + SR increased significantly, while in spite of increased expression of p-NF-κB ($p = 0.003$) in MI subjects, we didn’t see any statistical difference in iNOS expression at 21 days after MI induction in MI group comparing Sham group. Experience of SR after Sham surgery didn’t affect NF-κB expression in rats but it caused overexpression of iNOS ($p = 0.014$) in comparison with Sham subjects. When double obtained images of p-NF-κB and iNOS were merged together, it confirmed co-localization of p-NF-κB and iNOS positive cells in MI + SR group as compared to other experimental groups ($p < 0.05$).

3.9. Histological study

Figure 7 shows the results for evaluation of myocardial hypertrophy by H&E staining in which a significant increase in diameter of myocytes is seen in MI group and MI + SR animals as compared to Sham group ($p < 0.001$), and this increase was more obvious in MI + SR group ($p < 0.001$) in comparison with MI subjects. This histological analysis of myocardial hypertrophy also revealed a significant difference between MI and Sham + SR groups ($p = 0.016$) but there was no difference between Sham group and Sham + SR subjects ($p = 0.98$).

4. Discussion

Post-MI ventricular remodeling occurs in the myocardium remote from the infarct as well as the myocardium surrounding the infarcted tissue (border zone) to preserve cardiac function and limit progressive loss of cardiomyocytes during and after MI (Le et al., 2012). However, a variety of adaptive and maladaptive stimuli play critical roles in its modulation and may deteriorate LV contractile function which finally results in long-standing cardiac hypertrophy and HF (Durgan et al., 2010). Disturbed sleep or sleep loss due to vocational or lifestyle changes following MI is a common problem that may affect many physiological processes involved in LV remodeling (Bah et al., 2010). In the current study, we investigated whether the non-infarcted myocardium exhibits variations in its function/dysfunction in a rat model of chronic SR after MI through measures
of functional, biochemical, genetical and histopathological changes. Our results highlighted deteriorating effects of post-MI SR on NO production and oxidative stress, echocardiographic indexes, UPS system, immunohistochemical and histological parameters.

Literature data show a higher incidence of morbidity and mortality after induction of MI by LAD ligation, reaching a mortality of about 27.67–50% (Lobo Filho et al., 2011). In our study, we showed a mortality rate of 20% in MI subjects. By preventing trauma to the lungs as well as postoperative antibiotic therapy and rapid closure of the chest after LAD ligation, we observed that as well as 10% of subjects from MI group which died at first 24 h after MI induction, 30% of animals from MI + SR group died during the experience of SR; so, we had to replace these animals to get proportional sample size for our study. It seems that the higher mortality of MI + SR animals as compared to MI subjects is related to stress response caused by of SR-MSP method that may induce increased serum corticosterone in animals placed on these platforms (Carvalho et al., 2014). Alterations of corticosterone levels due to the lack of REM sleep and/or to the stress inherent of the technique itself were the main concern of our previous studies and we have shown that in rats which were placed in large multiple platforms (MLP), there was no larger hormone responses than rats which were subjected to MSP (data are not published). Herein, both Sham + SR and MI + SR subjects displayed a distinct increase in corticosterone levels compared to Sham and MI groups, confirming our SR paradigm could be as an intensive for stress outcome of REM sleep loss.

Chronic SR is often associated with body weight loss (Koban, 2009) and in the present study, this trend was confirmed in SR rats which started to gain weight after SR paradigm accomplishment. Of particular interest of our laboratory was to investigate about hyperphagia occurrence when SR is held to 6 days. In this regard, at the time of acclimation, the rats’ food intake was measured daily and the obtained average amount of ad libitum food consumption was 45 ± 2.1 g/day for each rat. Then, we measured accurate food intake of each rat by collecting spilled crumbs from the surrounding water, separating from waste, and drying them during 21 days post-surgery and found a substantial increase in food consumption of each sleep restricted animal from 1st day (41 ± 5.03 g/day for each rat) to 21st day after surgery (68 ± 3.4 g/day for each rat); i.e. this hyperphagia emerged only 4 days after experience of SR and it

Figure 7. Histological analysis of myocardial hypertrophy 21 days after surgery using one-way ANOVA (n=5). Transverse sections of the hearts which were stained with H&E staining to detect myocytes hypertrophy by light microscope (×100). Scale bar: 74 μm (left side). Quantification of myocardial hypertrophy: Experience of MI merely or with sleep restriction increased diameter of myocytes 21 days post-surgery (right side). MI: myocardial infarction, SR: sleep restriction. *p<0.05 compared to sham group $p<0.05 compared to MI group #p<0.05 compared to sham+SR group.
continued till the end of our experimental protocol. Moreover, MI rats developed a better coping strategy to deal with the stress of MI since they didn’t experience SR and had a proper weight gaining. Perhaps the enforcement of Sham + SR and MI + SR subjects to maintain wakefulness and feelings of stress results in high sympathetic tone that stimulates their metabolism compelling them to consume more (Koban et al., 2008).

Typical changes in the ECG including ST segment elevation and new Q-wave are important tools for detecting MI, but due to lower sensitivity of ECG, all of these changes may not initially present after MI and the need for use of cardiac markers for the diagnosis of MI seems to be crucial (Al-Hadi & Fox, 2009). In the present study, we confirmed successful constriction of LAD by ST elevation immediately after ligation and cyanosis of the affected myocardium; and in continue, we detected CK-MB levels within 24 h post-MI (CK-MB levels usually return to baseline levels after 24–48 h post-MI (Gutiérrez et al., 2009). The results revealed that animals with a total concentration of CK-MB more than twice the upper limit of normal (Gutiérrez et al., 2009) could be considered as MI subjects. Moreover, to evaluate cardiac function after MI induction, we used a model of permanent ligation of LAD in rat which is fundamentally different from ischemia/reperfusion injury. In the context of objectives and pathophysiological relevance, the infarct size following reperfusion can be modulated by factors affecting myocardial salvage, but 24 h after permanent ligation of LAD, the infarct area is fixed which affects cardiac function by infarct healing, scar formation, development of LV dilatation, cardiac hypertrophy and ventricular remodeling (van Zuylen et al., 2015).

To obtain more accurate measurements of these cardiac changes after MI and to ascertain about the accuracy of LAD ligation (Azar et al., 2014), we used an M-mode echocardiography that showed clear morphological changes like increase in LVDs and LVDd as well as functional changes including decrease in FS and EF in MI group as compared to Sham and Sham + SR groups at 21 days post infarction. Although ventricular enlargement and hypertrophy, reduced EF and FS are the main predictors for the MI prognosis (Murdoch et al., 2006), it might be argued that the major determinants of its prognosis are the factors leading to maladaptive remodeling (Cohn et al., 2000). This study established a clear link between SR after MI and the extent of LV dysfunction. We observed consistent associations of SR with echocardiographic markers of LV dimension and dysfunction. It seems reduced FS occurred, in part, as a result of progressive ventricular contractile dysfunction in MI + SR subjects (Cohn et al., 2000). Moreover, our observations regarding reduced EF in these animals reveal that experience of SR after MI by its related mechanisms may increase wall stress which leads to further dilation of the heart, greater increases in LV diastolic and systolic volumes and finally progress toward overt chronic HF (Cohn et al., 2000).

Further to this, the current study demonstrated an increased heart/body weight ratio in MI + SR rats in comparison with Sham and MI groups, suggesting that SR may decrease resistance to adverse remodeling and delayed global compensatory (reactive) ventricular hypertrophy of the heart after MI that is associated with its poor prognosis (Cohn et al., 2000). However, an increase in the heart/body weight ratio may not reflect the real increase in cardiac mass and its use as a marker of the degree of myocardial hypertrophy due to the alterations in either measure is problematic (Wallen et al., 2000). Although the total heart weight was lower in Sham + SR rats than in other experimental groups, our observation of lower heart/body weight ratio with normal echocardiographic findings of LV volume and function in these subjects may support the concept that this disproportionate decrease is associated with higher weight of sleep restricted animals at 21 days after Sham surgery.

Although an exact picture of all the molecular mechanisms involved in LV remodeling after MI is less clear, a key feature of this process is believed to be the fundamental role of myocytes as regards of their contractile activity and numeric contribution to heart mass (Cohn et al., 2000). Protein turnover by UPS for the survival of cardiac cells is a dynamic and regulated process that alterations in this protein quality control system are correlated with pathophysiology of MI (Shishido et al., 2008); i.e. progressive degradation of existing proteins by UPS and also synthesis of new contractile proteins in the myocytes may convert gene expression profile that consequently leads to pathologic hypertrophy and
alterations of the contractile apparatus (Adams et al., 2007). Although the specificity of the UPS system is
thought to rely on the E3 ubiquitin ligases as well as
proteasome function, it is said that ubiquitination of
proteins by E3 ligases (MaFBx and MuRF) has indepen-
dent and important roles than the proteasome
(Mearini et al., 2008). These ligases are highly
expressed following cardiac ischemia to modulate
cardiac function in response to different stresses
(Han et al., 2015). We found that SR significantly
increased MuRF1 and MaFBx levels following MI
compared to control MI suggesting that it might be
increased to inhibit LV hypertrophy (Conraads et al.,
2010; Willis et al., 2010; Li et al., 2011). However, this
enhanced ubiquitin ligases expression might not be
necessarily cardioprotective because their regulation
seems to be multifactorial in nature and possibly the
other biochemical mechanisms are involved (Willis
et al., 2010). DUBs such as A20 can conversely
remove ubiquitin chains from cellular proteins and
protect the infarcted heart against maladaptive
remodeling, progressive decompensation and myo-
cardial hypertrophy since they inhibit NF-κB signaling
(Düwel et al., 2009). Given the established
consequences of SR on cardiovascular function, it is
also pertinent to further consider its impact on the
expression of E3 ligases and DUBs since these all
directly affect maintenance of muscle mass (Anafi
et al., 2013). In the present study, we observed down-
regulation of A20 as well as overexpression of
MaFBx and MuRF1 in animals which experienced
SR after MI. Interestingly, mRNA expression of these
all three genes was upregulated in the heart of MI
subjects. The difference concerning A20 expression
in MI and MI + SR groups could be due to their
difference in activation of inflammatory processes
after MI. We previously observed remarkable upre-
gulation and production of pro-inflammatory cyto-
kines such as TNF-α 24 h post-MI which decreased
noticeably at 21 days after MI induction. In addition,
experience of SR either in Sham + SR group or in MI
+ SR rats caused a significant increase in activation of
TNF-α at the end of 21st day (data are not pub-
lished). It seems that such inflammatory response
and subsequent myocardial damage were suppressed
by A20 overexpression which is accompanied by
MaFBx and MuRF1 upregulation to inhibit LV dys-
function in MI subjects. Conversely, upregulation of
TNF-α in response to SR after MI may either
overexpress MaFBx and MuRF1 genes or inhibit
A20 expression, resulting in an imbalance between
E3 ligases and DUBs that in turn contributes to NF-
κB activation and cardiac hypertrophy (Usui et al.,
2011). It has been reported that A20 overexpression
may contribute to blockage of inflammatory
responses through NF-κB inactivation (Li et al.,
2007). In the current investigation, we also found
that A20 expression is higher in the Sham group with
SR compared to Sham alone indicating that A20
overexpression in Sham + SR subjects would attenu-
ate inflammatory responses mediated by SR experi-
ence (Mullington et al., 2010). Although we reported
herein that TNF-α overexpressed in Sham + SR
animals, it seems that TNF-α-dependent NF-κB activa-
tion requires several interrelated pathways (Kwon
et al., 2004) which may lessen its expression in spite
of increased levels of TNF-α. Thus, further studies
are needed to elucidate these specific pathways.

Emerging evidence suggests that NO acts as a
ubiquitous modulator of cardiac function and it is
noted that NO-mediated protection against adverse
LV remodeling attributes to inhibition of NF-κB
signaling after MI (Yin et al., 2008). However,
such a cardioprotective effect for the reason that it
may overexpress endothelial nitric oxide synthase
(eNOS) could be suppressed by overexpression of
inducible nitric oxide synthase (iNOS) induced
upon tissue ischemia and pro-inflammatory stimu-
lations (Yin et al., 2008) which correlates positively
with the generation of oxygen-free radicals as well
as overproduction of NO and ultimately leads to
LV dysfunction (Sun et al., 2009). In the present
study, an increase in iNOS expression and a
decrease in eNOS expression were observed in MI
+ SR group, when compared with the Sham sub-
jects. Furthermore, upregulation of eNOS was
detected in MI subjects as compared to Sham
group, using RT-PCR technique. Insignificant
iNOS expression was observed in MI group com-
paring to Sham rats, despite it was particularly high
in the Sham + SR group. Furthermore, we looked
on more details regarding the iNOS expression in
all experimental subjects by immunofluorescent mea-
suring and found that its results are in accordance
with our findings related to gene expression of
iNOS. Although we didn’t see any significant dif-
ference of NO serum concentration between experi-
mental groups at 21st day post-surgery, NO
production at 8th day post-surgery was found to be statistically high after SR experience in both Sham + SR and MI + SR groups. It seems that SR by itself significantly has caused activation of pro-inflammatory cytokines that in turn induce iNOS for producing larger amounts of NO during the first 8 days after Sham and MI surgery. Moreover, it is reported that NO bioactivity and decreased expression of eNOS related to increased oxidative stress rather than increased amount of NO and such thing may affect MI prognosis (Pacher et al., 2005); so, we can argue that in the current study, loss of NO bioactivity and/or at least downregulation of eNOS due to SR in MI + SR group contribute to their cardiac dysfunction and HF. Despite this, since eNOS requires to be activated, translocated for producing NO and iNOS constitutively forms NO depending on its level of expression, it should be noted that levels of NOS expression don’t necessarily predict the levels of NO (Sun, 2009). Accordingly, we observed that in spite of eNOS upregulation in MI group, serum concentration of NO at 8 days after MI induction was significantly decreased as compared to MI + SR subject.

Oxidative stress secondary to MI is the main process involved in LV remodeling which is the consequence of an imbalance between reactive oxygen and nitrogen species (RONS) production and/or impaired antioxidant defense (Ansley & Wang, 2013). Herein, 24 h after MI, we observed increased levels of NO and MDA. We also revealed that MDA levels significantly increased 8 days after surgery in animals which experienced SR and these amounts had been remained at high levels at 21st day after surgery as compared to MI and Sham groups; these findings suggest that SR may cause ROS to remain at high levels in a site-specific manner for development of LV hypertrophy (Maulik & Kumar, 2012). It is plausible that increased myocardial oxidative stress in MI + SR rats may activate NF-kB which in turn leads to activation of inflammatory processes and subsequent hypertrophy of cardiomyocytes. Herein, overexpression of NF-kB seems to be as an explanation for the observed hypertrophy in MI and MI + SR groups. However, we should consider that myocardial hypertrophy in animals which experienced SR after MI was significantly obvious which was confirmed by co-localization of p-NF-kB and iNOS in these subjects. It also seems that overexpression of iNOS has caused NO to be produced considerably and its coexistence with ROS contributes to aggregated function of the heart due to further myocardial hypertrophy in MI + SR group.

Finally, it should be noted that although in the current study we compared echocardiographic and histologic data of MI and MI + SR groups with Sham subjects at 21 days post-surgery, one of the significant limitations of this study was that no baseline measurements of LV function or histologic measurements of infarct size were measured which certainly would cause one does not know if these animals started out with similar infarcts or not.

Taken together, our results add to the knowledge regarding the importance of sleep quality in the process of recovery after MI. Our data suggest that SR after MI leads to an enlargement of the heart within 21 days, marked by an increase in oxidative stress and NO production as well as an imbalance in UPS that ultimately results in cardiac dysfunction and HF. It should be noted that we are in the first steps of our research to know possible mechanisms due to post-MI sleep loss that may aggregate MI prognosis and it is our future purpose to consider effective treatments for alleviating sleep disturbances after MI which would prevent LV adverse remodeling.

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Declaration of interest
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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