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Acute sleep deprivation induces cardioprotection against ischemia/reperfusion injury through reducing inflammatory responses: the role of central GABA-A receptors

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Abstract. Sleep is considered as a physiological regulator in the body. Gamma-aminobutyric acid (GABA) is a neurotransmitter that modulates sleep and affects cardiac functions. We evaluated effects of acute sleep deprivation (SD) on cardiac hemodynamic parameters, expression of pro-inflammatory cytokines, and Heat shock protein (*Hsp70*), serum level of lactate dehydrogenase (LDH) and prooxidant/antioxidant balance (PAB). Male Wistar rats were bilaterally cannulated in the central nucleus of amygdala (CeA) and saline or bicuculline was injected 24 hours prior to induction of 30 minute ischemia following 120 minute reperfusion. Forty-eight animals were randomly divided into four groups: Control (CONT), bicuculline (BIC), acute SD and bicuculline + acute sleep deprivation (BIC+SD). Animals in SD and BIC+SD groups were put in an aquarium for inducing sleep deprivation. SD attenuated LDH, pro-inflammatory cytokines and PAB; improved cardiac hemodynamic parameters and increased *Hsp70* in non-infarcted area as compared to CONT. Administration of bicuculline increased LDH, pro-inflammatory cytokines and PAB, reduced cardiac hemodynamic parameters and *Hsp70* as compared to CONT. Furthermore, bicuculline administration prior to acute sleep induction decreased SD effects on LDH, PAB, *Hsp70*, cardiac hemodynamic parameters and pro-inflammatory cytokines. Induction of SD prior to ischemia/reperfusion induces cardioprotection through suppressing inflammatory responses.

Key words: Acute sleep deprivation — Cardioprotection — GABA — Inflammatory responses — Lactate dehydrogenase

Abbreviations: cDNA, complementary DNA; CeA, central nucleus of amygdala; GABA, gamma-aminobutyric acid; HPRT, hypoxanthine phosphoribosyl transferase-encoding gene; Hsp, heat shock protein; IL, interleukin; IR, ischemia-reperfusion; LDH, lactate dehydrogenase; LVEDP, left ventricular end diastolic pressure; LVSP, left ventricular systolic pressure; MI, myocardial infarction; MIRI, myocardial IR injury; NF-κB, nuclear factor-κB; nqRT-PCR, quantitative reverse transcription polymerase chain reaction; NTS, nucleus tractus solitarius; PAB, prooxidant/ antioxidant balance; PCR, polymerase chain reaction; ROS, reactive oxygen species; SD, sleep deprivation; TNF-α, tumor necrosis factor α; UPS, ubiquitin proteasome system.

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Introduction

Cardiac reperfusion after an acute myocardial infarction (MI) often results in death of previously viable myocytes, a phenomenon termed as "Myocardial IR injury" (MIRI).

Subsequent to necrotic cardiomyocyte death, an inflammatory cascade is activated and led to cardiac tissue damage (Ling et al. 2013). Sleep is a modulator of behaviour and physiological responses of the body and therefore, the duration and the quality of sleep can affect functioning of many organs. In this aspect, it has been reported that acute sleep deprivation shows protective effects (Edalatyzadeh et al. 2016; Pace et al. 2017). In the bidirectional crosstalk between sleep and the immune system, cytokines as 'sleep regulatory substance' play a prominent role in the homeostatic regulation of sleep in normal physiological conditions. However, experimental induction of sleep loss does not seem to alter circulating markers of inflammation (Dimitrov et al. 2015; Irwin et al. 2016). Gamma amino butyric acid (GABA) is a well-known regulatory neurotransmitter, its receptors are widely distributed in central nucleus of the amygdala (CeA) which modulates sleep (Liu et al. 2009; Chanana et al. 2016). GABA modulates cardiovascular functions by affecting central nervous system through nucleus tractus solitarius (NTS) (Avolio et al. 2014) and peripheral tissues. GABA has been shown to alter vascular tone and sympathetic activity in various tissue/organs like artery, kidney, mesenteric arterial bed, and the pulmonary artery in rabbit, rat and cat respectively (Lingeshwar et al. 2016). Evidence suggests that proinflammatory molecules released during autoimmune encephalomyelitis may affect central GABA activity (Sallam et al. 2016).

One of the central players in inflammatory signaling is the transcription factor nuclear factor-κB (NF-κB). Numerous reports have shown that NF-κB is activated after myocardial ischemia-reperfusion (IR) (Ling et al. 2013). NF-κB translocate to the nucleus after releasing from its inhibitory subunit IκBα, and causes transcriptional regulation of the expression of interleukins and cytokines. The NF-κB-mediated inflammatory cascade can be perpetuated through the ability of NF-κB targets, such as tumor necrosis factor α (TNF-α) and interleukins to further activate NF-κB signaling, as well as by the infiltration of neutrophils and other mononuclear inflammatory cells at sites of injury (Ling et al. 2013). Lactate dehydrogenase (LDH) is a marker of myocardial injury which increases after IR (Cheng et al. 2016). It has been shown that LDH level increases in cardiac tissue after chronic sleep deprivation (Jeddi et al. 2016). Several recent studies have suggested the role of exaggerated reactive oxygen species (ROS) production and suppressed endogenous antioxidant defense such as heat shock proteins (Hsp's) in the pathogenesis of myocardial IR injury (Rani et al. 2013). Hsp 70 inhibits cardiomyocyte necroptosis through repressing autophagy in myocardial IR injury (Rani et al. 2013). Additionally, it has been reported that *Hsp70* levels alters during sleep deprivation (Lapshina et al. 2010). The expression of *Hsp70* has been shown to markedly increase in patients with sleep apnea (Lavie et al. 2010).

Although many studies have evaluated the role of inflammation in mediating IR injury, but the cardio protective role of acute sleep deprivation (SD) in suppression of IR-induced inflammation has not yet been studied.

Material and Methods

Animals

Forty-eight male adult Wistar rats (250–300 g) were housed in the animal house of the physiology department providing 12 h light/dark cycle, temperature 23 ± 2°C, and unlimited access to food and water. The experimental protocols (Fig. 1) in this study were conformed to the Guidelines of the Care and Use of Laboratory Animals published by National Institutes of Health (NIH Publication No.85-23, revised 1996) and was further approved by the institutional ethical committee of Tehran University of Medical Sciences (Tehran, Iran). Acute sleep deprivasion was induced from 7:00 AM for 24 h.

Groups

The central nucleus of amygdala (CeA) was bilaterally cannulated in all animals. After 5 days recovery, saline or bicuculline (a GABA-A antagonist) was injected into CeA. 24 h after saline or bicuculline injection, all rats were underwent 30 min ischemia followed by 120 min reperfusion for induction of IR. Animals were randomly divided into four groups (animal/group n = 12) as below: (1) Control (CONT) group: saline was injected and 24 h later underwent for 30 min ischemia followed by 120 min reperfusion, (2) Bicuculline (BIC) group : bicuculline as GABA-A antagonist was administrated 24 h prior to 30 min ischemia followed by 120 min reperfusion, (3) Acute sleep deprived (SD) group : after administration of saline, animals were put in the aquarium by small plates for 24 h induction of acute sleep deprivation, and then subjected to 30 min ischemia followed by 120 min reperfusion, (4) Bicuculline + acute sleep deprivation (BIC+SD) group : bicuculline was injected Intra-CeA, then animals were put in aquarium by small plates for 24 h to induce acute sleep deprivation, then underwent for 30 min ischemia followed by 120 min reperfusion. At the end of reperfusion, blood samples were collected from anesthetic rat's heart. Then hearts were separated for genetic assessment.

Stereotaxic surgery and drugs

All rats were anesthetized by ketamine (50 mg/kg) and xylazine (5 mg/kg, *i.p.*), and then were placed in a stereotaxic frame (Stoelting, USA) and the stainless steel guide cannulas (23 gauge, 15 mm length) were bilaterally implanted into the CeA with following coordinates according to Paxinus

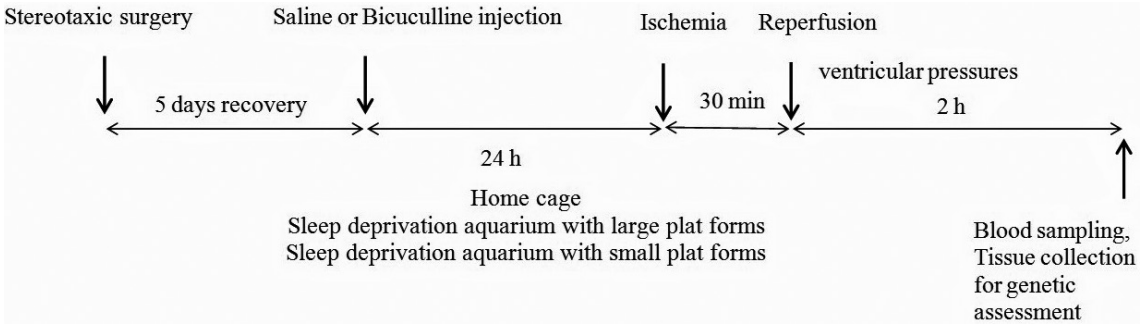

Figure 1. The experimental protocol.

and Whatson atlas (Anterior-Posterior: –2.8, Lateral: ± 4.6, Dorsal-Ventral: –8.1) (Paxinos et al. 2007; Riahi et al. 2013).

Bicuculline methiodide (Sigma Aldrich) 0.1 nmol/0.5 µl dissolved in normal saline. Although bicuculline methiodide is use for epilepsy induction, but this dosage that we administrated is not epileptic (Winn et al. 1980; Abbas 2015). Saline or bicuculline was injected 0.5 µl at each side intra-CeA.

Acute sleep deprivation induction

24-h sleep deprivation was induced by putting the animals in an 8 circular small platforms aquarium (125 × 44 × 44 cm and 6.5 cm in diameter) has been explained before (Parsa et al. 2016; Zagaar et al. 2016). The EEG recording confirmed the induction of sleep deprivation by multiple plate forms model (Arthaud et al. 2015).

Myocardial ischemia and reperfusion

All rats were anesthetized with thiopental (60 mg/kg, *i.p.*) and the left anterior descending coronary artery (LAD) was ligated from origin according to our previous studies (Imani et al. 2011; Azizi et al. 2015).

Blood sampling and Elisa assessment

Blood samples were collected from the heart at the end of experiment for biochemical analysis (LDH, TNF-α and prooxidant/antioxidant balance (PAB)). The samples were centrifuged at 7,000 rpm, 4°C, for 20 min; the serum was removed and stored at –70°C until biochemical analysis. Serum level of LDH, TNF-α, and PAB was measured by a commercial ELISA kit (Pars azmoon, Iran), according to the manufacturer's instructions.

Assessment of myocardial function

Changes of heart function were assessed by measuring of cardiac hemodynamic parameters. A small incision was made to the right of the midline in the neck. The right common carotid artery was exposed and cannulated with a PE50 catheter connected to the Power lab system through pressure transducer. Catheter was pushed down until it had reached the left ventricular lumen. Left ventricular systolic pressure (LVSP) and left ventricular end diastolic pressure (LVEDP) were all monitored and recorded by Powerlab data acquisition system (AD Instrument, Australia) (Azizi et al. 2015).

Real-time polymerase chain reaction (Real-Time PCR)

At the end of study, we removed the myocardial samples (n = 4), rinsed in PBS, frizzed in liquid nitrogen and stored at –80°C. Total RNA was extracted from frozen infarcted and non-infarcted area by using Trizol (Invitrogen, Carlsbad, CA). A PrimeScript RT reagent kit (Takara, Cat. RR037A) was used to provide complementary DNA (cDNA) as templates for quantitative reverse transcription polymerase chain reaction (qRT-PCR), for quantification of gene expression, we used Rotor-Gene 6000 (Qiagen). Real-Time PCR analysis was provided by the use of SYBER Permix Ex Taq (Takar, Cat.RR280L). The value for each sample was an average of two independent PCR measurements. Hypoxanthine phosphoribosyl transferase-encoding gene (HPRT) was used as normalized to control (Azizi et al. 2015). The specific primer sequences are listed in Table 1.

Statistical analysis

Statistical analyses were done by one way ANOVA followed by Tukey post *hoc* test, using SPSS software (Version 22, SPSS IBM, Chicago, IL). All data are shown as mean ± standard error of the mean (SEM) and $p < 0.05$ was considered statistically significant. Sample size was determined based on previous studies which used to obtain significant results (Azizi et al. 2013, 2015). None of the samples were excluded from the analysis. The animals were randomly allocated to experimental groups and no blinding was observed during the assessment of experimental outcomes.

Table1. The sequence of primers used in Real-Time PCR

Gene name	Primer sequence	PCR product size
Hsp70	R: 5'- TGAAGACCAGCACCGTGGA -3' F: 5'- TGGCGTCATTCCGTTCCTTC -3'	138
IL-6	R: 5'- AGCCAGAGTCATTCAGAGCAA-3' F: 5'- TTGGTCCTTAGCCACTCCTTC -3'	150
TNFα	R: 5'-ACCACGCTCTTCTGTCTACTGA-3' F: 5'-GGCTTGTCACTCGAGTTTTGAGA -3'	85
HPRT	R: 5'-GCAGGTCAGCAAAGAACTTATAGCC-3' F: 5'-CTCATGGACTGATTATGGACAGGAC-3'	123

Results

Effects of acute sleep deprivation on LDH level in serum

LDH was measured as a marker of myocardial injury. Our results showed that bicuculline administration in BIC group increased LDH level (5901.8 ± 487.8, 4016.8 ± 264.7; $p < 0.01$) as compared to CONT group. Acute sleep deprivation in SD group decreased LDH level (2601.8 ± 367.2; $p < 0.05$) as compared to CONT group. Bicuculline injection prior acute sleep deprivation in BIC+SD group increased LDH in serum (4490 ± 225.2, $p < 0.01$) as compared to SD group (Fig. 2).

Effects of acute sleep deprivation on PAB level in serum

Results shown in Fig. 3, explain that bicuculline administration in BIC group increased PAB level as compared to CONT group (13.41 ± 2.31, 2.67 ± 0.64; $p < 0.01$). Acute sleep deprivation in SD group decreased PAB level none significantly as compared to CONT group (1.94 ± 0.34). Bicuculline injection prior acute sleep deprivation in BIC+SD group increased PAB level in serum none significantly as compared to SD group (4.35 ± 0.35).

Assessment of cardiac hemodynamic parameters

To assessment the myocardial hemodynamic parameters, we evaluated left ventricular systolic and end diastolic pressure. Our results showed that administration of bicuculline in BIC group decreased LVSP (81 ± 3.5) and increased LVEDP (6.5 ± 0.3) as compared to CONT group (LVSP: 100.4 ± 1.6, LVEDP: 4 ± 0.6, $p < 0.05$). Acute sleep deprivation induction in SD group increased LVSP (121.3 ± 1.6) and declined LVEDP (1.3 ± 0.5) as compared to CONT (LVSP: $p < 0.05$, LVEDP: $p < 0.01$) and BIC group (LVSP: $p < 0.001$, LVEDP: $p < 0.01$). Injection of bicuculline prior to acute sleep dep-

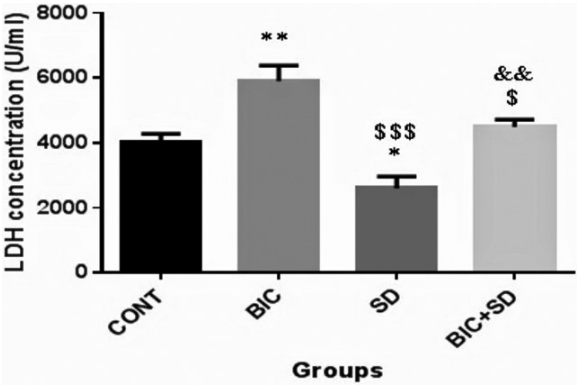

Figure 2. Lactate dehydrogenase (LDH) concentration in serum. All samples were analyzed in duplicate. Data are presented as mean ± SEM (animals/group $n = 6$). Groups: BIC, bicuculline; BIC+SD, bicuculline + acute sleep deprivation; CONT, ischemia-reperfusion; SD, acute sleep deprivation. * $p < 0.05$ and ** $p < 0.01$ *vs.* CONT group, $^{\$}$ $p < 0.05$ and $^{\$\$\$}$ $p < 0.001$ *vs.* BIC group, $^{\&\&}$ $p < 0.01$ *vs.* SD group.

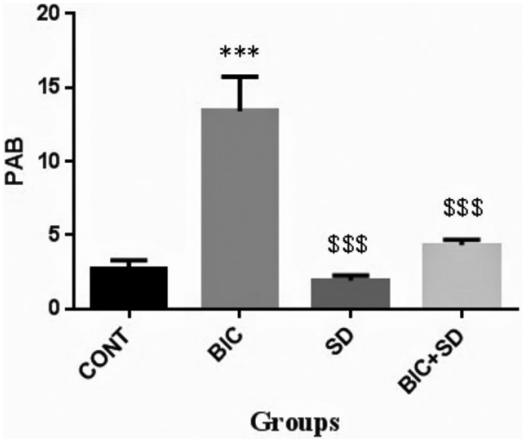

Figure 3. PAB concentration in serum. All samples were analyzed in duplicate. Data are presented as mean ± SEM (animals/group $n = 6$). *** $p < 0.01$ *vs.* CONT group, $^{\$\$\$}$ $p < 0.001$ *vs.* BIC group. PAB, prooxidant/antioxidant balance. For more abbreviations, see Fig. 2.

rivation in BIC+SD group decreased LVSP (102.7 ± 6) and increased LVEDP (3.6 ± 055) as compared to SD group ($p < 0.05$) (Fig. 4).

Effects of acute sleep deprivation on gene expression

To evaluate the effect of acute sleep deprivation on gene expression (*Hsp70, IL-6, TNF-α*) in heart tissue, we divided left ventricle into infarcted and non-infarcted area in all groups.

Effects of acute sleep deprivation on Hsp70 expression

Fig. 5A shows *Hsp70* expression in infarcted and non-infarcted area. Our results declare that *Hsp70* expression was decreased fallowing bicuculline injection in BIC group (0.9 ± 0.01, $p < 0.001$) as compared to CONT group. Acute sleep deprivation in SD group increased *Hsp70* expression (1.44 ± 0.02, $p < 0.001$) as compared to BIC group. induction of acute sleep deprivation after bicuculline administration in BIC+SD group increased *Hsp70* expression in infarcted area (1.15 ± 0.06, $p < 0.001$) as compared to BIC group.

Hsp70 expression is declined in non-infarcted area in BIC group (0.02 ± 0.01, $p < 0.001$) as compared to CONT group. Induction of acute sleep deprivation in SD group increased *Hsp70* expression (70 ± 0.26, $p < 0.001$) in non-infarcted area as compared to CONT group. Injection of bicuculline prior to acute sleep deprivation in BIC+SD group decreased elevated *Hsp70* expression induced by acute sleep deprivation in non-infarcted area (1.2 ± 0.24, $p < 0.001$) as compared to BIC group.

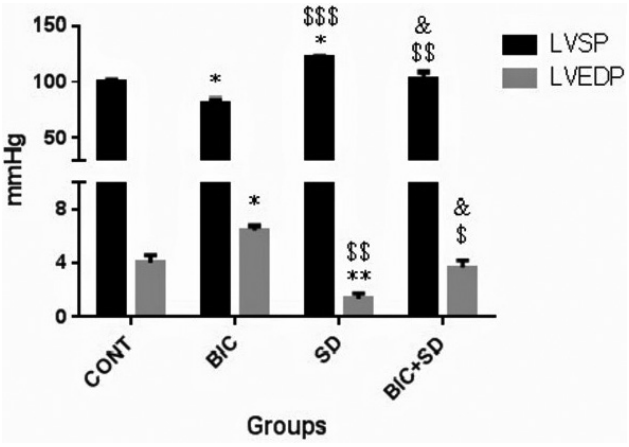

Figure 4. Left ventricular systolic pressure (LVSP) and left ventricular end diastolic pressure (LVEDP). * $p < 0.05$ and ** $p < 0.01$ *vs.* CONT group, $^{\$}$ $p < 0.05$, $^{\$\$}$ $p < 0.01$ and $^{\$\$\$}$ $p < 0.001$ *vs.* BIC group, $^{\&}$ $p < 0.05$ *vs.* SD group. For more abbreviations, see Fig. 2.

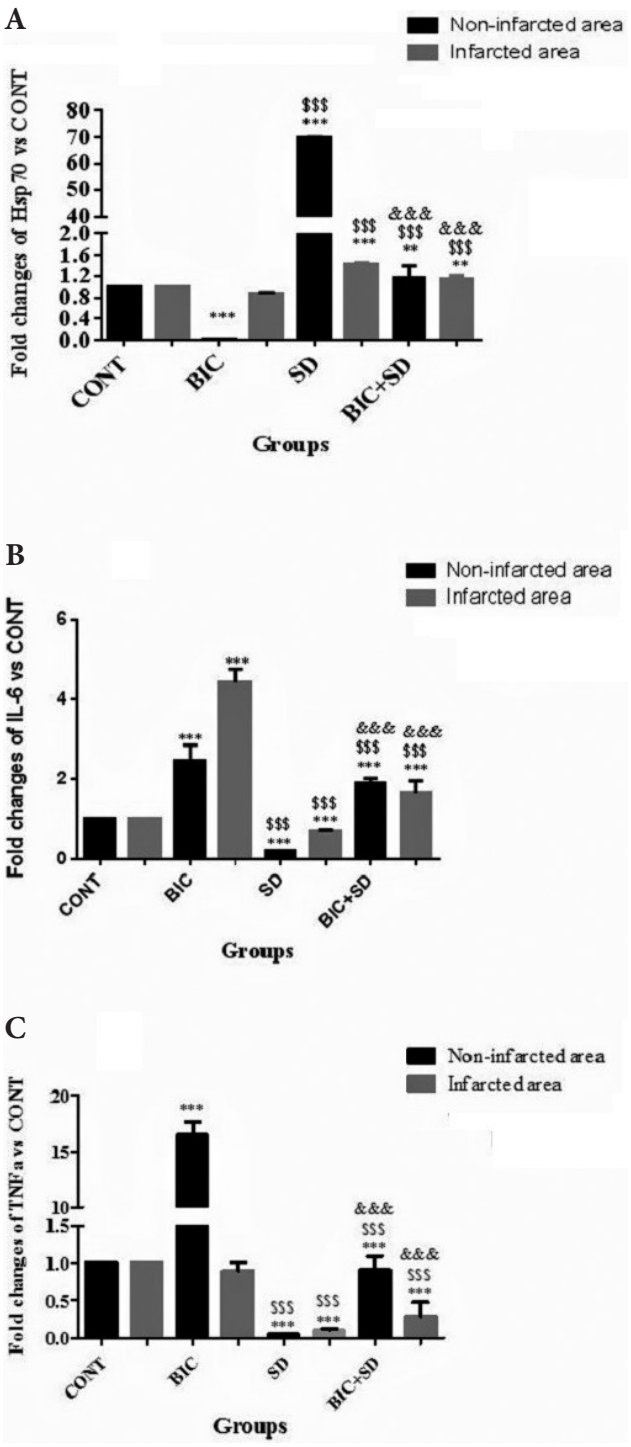

Figure 5. Fold of changes of *Hsp70* (**A**), *IL-6* (**B**) and *TNF-α* (**C**) mRNA expression *vs.* CONT group in infarcted area and non-infarcted area. All samples were analyzed in duplicate. Data are presented as mean ± SEM (animals/group $n = 5$). ** $p < 0.01$ and *** $p < 0.001$ *vs.* CONT group, $^{\$\$\$}$ $p < 0.001$ *vs.* BIC group, $^{\&\&\&}$ $p < 0.001$ *vs.* SD group. Hsp, heat shock protein; IL, interleukin; TNF-α, tumor necrosis factor α. For more abbreviations, see Fig. 2.

Effects of acute sleep deprivation on IL-6 expression

As seen in Fig. 5B, *IL-6* expression in infarcted area of BIC group increased (4.45 ± 0.34, $p < 0.001$) as compared to CONT group. *IL-6* expression in SD group is decreased (0.7 ± 0.02, $p < 0.001$) as compared to CONT group. Injection of bicuculline into central nucleus of amygdala before acute sleep deprivation induction in BIC+SD group increased *IL-6* expression (1.7 ± 0.3, $p < 0.001$) as compared to SD group. *Il-6* expression in non-infarcted area increased in BIC group (2.5 ± 0.4, $p < 0.001$) as compared to CONT group. Acute sleep deprivation in SD group decreased *IL-6* expression in non-infarcted area (0.2 ± 0.01, $p < 0.001$) as compared to CONT group. Blocking GABA receptors in BIC+SD group increased *IL-6* expression in non-infarcted area (2 ± 0.12, $p < 0.01$) as compared to SD group.

Effects of acute sleep deprivation on TNF-α expression

Results of RT PCR studies revealed that the *TNF-α* expression in non-infarcted area was increased in BIC group (16.43 ± 1.15, $p < 0.001$) as compared to CONT group. Acute sleep deprivation in SD group reduced *TNF-α* expression in non-infarcted area (0.05 ± 0.009, $p < 0.001$) as compared to CONT group and bicuculline administration prior to acute sleep deprivation induction in BIC+SD group declined *TNF-α* expression in non-infarcted area (0.91 ± 0.1, $p < 0.001$) as compared to SD group. *TNF-α* mRNA expression in infarcted area following blocking GABA receptors in BIC group slightly decreased (0.9 ± 0.17) and induction of acute sleep deprivation in SD group declined (0.1 ± 0.02, $p < 0.001$) as compared to CONT group. Blocking GABA receptors prior to inducing acute sleep deprivation in BIC+SD group increased TNF-α expression in infarcted area (0.29 ± 0.18, $p < 0.001$) *TNF-α* expression as compared to SD group (Fig. 5C).

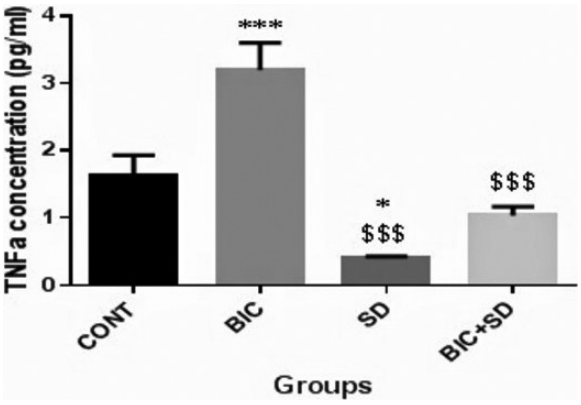

Figure 6. Concentration of TNF-α in serum. All samples were analyzed in duplicate. Data are presented as mean ± SEM (animals/group $n = 6$). * $p < 0.05$ and *** $p < 0.001$ *vs.* CONT group, $^{\$\$\$}$ $p < 0.001$ *vs.* BIC group. For more abbreviations, see Fig. 2.

Effects of acute sleep deprivation on TNF-α level in serum

It is illustrated in Fig. 6 that bicuculline administration in BIC group increased *TNF-α* level (3.2 ± 0.4, 1.62 ± 0.31, $p < 0.001$) as compared to CONT group. Acute sleep deprivation in SD group decreased *TNF-α* level (0.399 ± 0.03, $p < 0.05$) as compared to CONT group. Bicuculline injection prior to acute sleep deprivation induction in BIC+SD group increased *TNF-α* in serum not significantly as compared to SD group (1.04 ± 0.13).

Discussion

In this study, for the first time to our knowledge, we evaluated the effects of acute sleep deprivation on inflammation induced following myocardial ischemia/reperfusion. To assess the myocardial injury, LDH was measured and our results revealed that acute sleep deprivation decreased LDH levels in serum and showed cardiac protective effect against IR injury. Blocking GABA-A receptors increased this marker of myocardial injury and administration of bicuculline prior to SD attenuated the protective effects of SD. In this regard, Erikson et al. (2017) demonstrated a considerable rise in LDH levels following myocardial IR, however these levels were lower as compared to chronic sleep deprivation (Periasamy et al. 2015; Erikson et al. 2017). In the current study, the myocardial hemodynamic parameters were studied to reflect cardiac functions. We observed that SD increased left ventricular systolic and end diastolic pressure, while bicuculline administration prior SD induction reduced the beneficial effects of SD in this aspect. GABA is involved in regulating cardiac function and GABAergic projections are extended from central nervous system to NTS, the nucleus which has been shown to modulate cardiac performance. GABA receptors are up-regulated during wakefulness which positively modulate the endogenous agonist effects and enhance GABAergic currents (Matsuki et al. 2015), Furthermore, promotes GABA stabilizing and regulatory roles for hemodynamic parameters of the heart (Ma et al. 2015; Hussain et al. 2016; Sallam et al. 2016). Since we aimed to evaluate possible effects of SD in remote areas along with infarcted region, mRNA expression for *Hsp70*, *IL-6* and *TNF-α* expression was investigated in both infarcted and non-infarcted areas of heart. Our results showed that *Hsp70* expression in both infarcted and non-infarcted area increased after SD induction and bicuculline administration prior to SD reduced the expression of *Hsp70*. *Hsp70* is known to act as a molecular chaperone to maintain cellular homeostasis and inhibit protein aggregation (Bernardo et al. 2016). It has

been shown that sleep deprivation for 5 hours produces an elevation of the brain temperature and a significant increase of the muscle tone during the active wakefulness. An increase in brain temperature during sleep deprivation seems to be due to a rise in heat production which would be a stress and lead to activate heat shock proteins (Lapshina et al. 2010). Numerous studies have demonstrated the ability of *Hsp70* to attenuate the IR-injury markers of cardiac pathology. *Hsp70* has various roles in protein quality control including protein folding/refolding, the ubiquitin proteasome system (UPS) and autophagy. *Hsp70* is also known to regulate hypertrophic signaling and the inflammatory response after a cardiac insult (Bernardo et al. 2016). Therefore, up-regulation of *Hsp70* following SD can be indicative of SD-induced cardioprotection. Pro-inflammatory cytokines contribute to cardiac dysfunction *via* (i) increasing the generation of toxic oxygen species, (ii) alterations in cardiomyocyte calcium and mitochondrial homeostasis, and (iii) suppression of central sympathetic tone and cardiac baroreflex mechanisms (Sallam et al. 2016). It has been shown that TNF-α and IL-6 are involved in pathogenesis of cardiovascular diseases (Kleinbongard et al. 2010; Consortium 2012). We found that bicuculline increased the mRNA expression of TNF-α in non-infarcted area and IL-6 in both infarcted and non-infarcted areas which may suggest bicuculline-created inflammation. Moreover, the elevated IL-6 can be attributed to the increase in T-cells in the infarcted area through infiltration during reperfusion period (McGinnis et al. 2015). In non-infarcted area, high expression of TNF-α may be the result of activation inflammatory pathways which may induce IL-6 too. In contrast, SD reduced the expression of these proinflammatory cytokines. In this regard, Irwin and his colleague reported that sleep disturbance can influence the levels of IL-6 and TNF-α (Irwin et al. 2016), which is difference with our results that could be because of the duration of sleep deprivation.

Conclusion

GABA shows cardioprotective effects through different mechanisms. In this study, we assessed inflammation as one probable mechanism. Based on our results, *TNF-α* and IL-6 are down- regulated during acute sleep deprivation and blocking GABA-A receptors in central nucleus of amygdala upregulates these pro-inflammatory cytokines. On the other hand, *Hsp70* which has protective effects is upregulated following acute sleep deprivation. Additionally myocardial injury is increased due to bicuculline injection into central nucleus of amygdala.

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Conflict of interest. The authors of this study have no conflict of interest.

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