RETRACTED ARTICLE: LncRNA TUG1 protects against cardiomyocyte ischaemia reperfusion injury by inhibiting HMGB1

Hanyu Shi, Zhenhua Dong and Haiqing Gao

ABSTRACT
The aim of this study was to investigate whether LncRNA TUG1 could mediate the progression of ischaemia-reperfusion injury following acute myocardial infarction. Mouse cardiomyocytes HL-1 cells were subjected to oxygen glucose deprivation followed by reperfusion (OGD/R) to induce myocardial I/R injury. The expression of TUG1 was detected by real-time PCR. Overexpression or down expression of TUG1 was performed in mouse HL-1 cardiomyocytes. The myocardial cell viability and apoptosis were respectively detected. In addition, the expression levels of inflammatory factors, apoptosis-related proteins and HMGB1 proteins were detected. Besides, an inhibitor of HMGB1 was used to treat cells to verify the relationship between TUG1 and HMGB1 protein. The expression of TUG1 was significantly up-regulated in OGD/R-induced myocardial HL-1 cells. The overexpression of TUG1-induced inflammation and apoptosis in OGD/R-induced myocardial HL-1 cells. Knock down of TUG1 protected OGD/R-induced myocardial I/R injury by inhibiting HMGB1 expression. Suppression of LncRNA TUG1 may prevent myocardial I/R injury following acute myocardial infarction via inhibiting HMGB1 expression.

Introduction
Myocardial ischaemic reperfusion injury (MIRI) is a phenomenon that myocardial ischaemic injury is aggravated when ischaemic myocardial restores blood [1]. The incidence of coronary heart disease with acute myocardial infarction is increased worldwide now [2]. In the process of blood flow reperfusion, however, it leads to a large number of inflammatory cells aggregation in the myocardium of original ischaemia, serious damage to vascular endothelial function, obvious metabolic dysfunction, arrhythmia, myocardial apoptosis [3–5]. It is the main cause of serious cardiac complications even death after coronary revascularization and heart transplantation [6,7]. Therefore, it has important clinical significance of reducing the injury of myocardial tissue after myocardial ischaemia and reperfusion, which can significantly reduce post-operative complications and mortality.

High mobility group box 1 (HMGB1), a ubiquitous and abundant nuclear protein, has participated in the pathophysiological process of MIRI [8–10]. HMGB1 was significantly increased in the damaged myocardial tissue during the ischaemia reperfusion [11–13]. HMGB1 was binding to the toll-like receptor-4 (TLR-4) ligand and activation the membrane surface MyD88, and further lead to NF-κB excessive activation, which induced inflammatory cytokines interleukin-6 (IL-6) and tumour necrotic factor-α (TNF-α) huge release to produce severe inflammatory response [14–16].

Long non-coding RNAs (lncRNAs) are non-protein-coding transcripts longer than 200 nucleotides, and exert their physiological and pathological functions through their interactions with genomic DNA, miRNAs, mRNAs and proteins [17,18]. At present, several studies have revealed the important roles of dysregulated lncRNA profiles in the pathogenesis of ischaemic injury in various organs including liver, heart and brain [19,20]. For instance, suppression of lncRNA KCNQ1QT1 may prevent myocardial I/R injury following acute myocardial infarction via regulating AdipoR1 and involving in p38 MAPK/NF-κB signal pathway [21]. LncRNA ROR significantly promoted H/R-induced myocardial injury via stimulating release of LDH, MDA, SOD, and GSH-PX [22]. LncRNA MALAT1 was expressed at a high level in patients with acute myocardial infarction and was closely associated with the pathogenesis of myocardial I/R injury [23].

Taurine-upregulated gene 1 (TUG1) was firstly reported to be upregulated in exposure to the treatment of taurine in mouse retinal cells [24]. TUG1 has been proved to act as a tumour suppressor or oncogene in various cancers [25–27]. However, whether dysregulation of lncRNA TUG1 has protective effects against myocardial ischaemia/reperfusion injury following acute myocardial infarction has not been fully investigated.

In present study, we used the mouse cardiomyocytes HL-1 cells under condition of oxygen glucose deprivation followed...
by reperfusion (OGD/R) to induce myocardial I/R injury in vitro. The effects of up-regulation or down-regulation of TUG1 on cell viability, inflammatory response and cell apoptosis were investigated. Moreover, the regulatory relationship between TUG1 and HMGB1 expression was also determined. Our study aimed to investigate the protective effects of TUG1 on the cardiomyocyte ischemia reperfusion injury and its underlying molecular mechanisms.

Materials and methods

Cell lines

The mouse cardiomyocytes HL-1 cell line was purchased from ATCC. HL-1 cells were cultured in Claycomb medium containing 10% foetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 mM norepinephrine and 2 mM L-Glutamine at 37°C in an incubator with 95% air and 5% CO2.

Myocardial ischemia reperfusion injury model

HL-1 cells processed a condition of oxygen glucose deprivation followed by reperfusion (OGD/R) to induce myocardial ischaemia reperfusion injury model in vitro. In brief, the cells were incubated for 24 h in 96-well plates, then cultured with glucose-free Claycomb medium and incubated in an oxygen-free atmosphere (95% N2 and 5% CO2, 37°C) for 4 h. Following, the cells were incubated in normal culture medium (4.5 mg/mL glucose) and normal atmosphere (95% air and 5% CO2, 37°C) for another 24 h.

Cell transfection

Vector pc-TUG1 for overexpression of TUG1 was constructed by inserting the coding oligonucleotides of TUG1 into pCDNA3.1 vector (Invitrogen, Shanghai, China). pcDNA3.1 vector (pc-NC) was considered as the control. Small interference RNAs (siRNAs) targeting TUG1 (si-TUG1) and its control siRNAs (si-NC) were designed and synthesized by Invitrogen (Shanghai, China). OGD/R-induced I/R injury model HL-1 cells were cultured in the plates for 24 h and transfected with pc-TUG1, pc-NC, si-TUG, and si-NC using lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA). The cells were incubated 48 h after transfection before next experiments.

Cell treatment

To determine the relationship between TUG1 and HMGB1 protein in preventing OGD/R-induced myocardial injury, the OGD/R-induced myocardial HL-1 cells were exposed to 10 µM of HMGB1 inhibitor sodium butyrate (Aladdin, Shanghai, China) during the OGD/R procedures.

MTT assay

Cells were seeded into a 96-well plate with a density of 1 × 10⁴ cells/mL. Cell viability was examined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT) assay. After 24, 48, 72 and 96 h of transfection, 10 µL MTT solution (5 mg/mL, Beyotime, Shanghai, China) was added into each well and cultured for 4 h. The absorbance at 490 nm was measured after 150 µL dimethylsulphoxide (DMSO, Sigma) added and shaken for 10 min. The curve of cell proliferation was then drawn and the proliferation efficiency was examined. The experiments were repeated three times independently.

Cell apoptosis assay

Cell apoptosis was determined using an Annexin-V fluorescein isothiocyanate and propidium iodide (FITC/PI) apoptosis detection kit (Life Technologies, Waltham, MA) with flow cytometry in accordance with the manufacturer’s instruction. Briefly, 1 × 10⁶ cells were harvested after 48 h transfection and stained using Annexin-V FITC/PI. Cell samples were analysed using flow cytometry and a FACScan within 1 h.

RNA isolation and qRT-PCR

Total RNA was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The concentrations and purity of all RNA samples were detected by NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Houston, TX). Green miRNA qRT-PCR Kit (Invitrogen, Carlsbad, CA) was used to synthesize specific cDNA and perform qRT-PCR, which was analysed with the DNA Engine Opticon 2 Real-Time Cycler (MJ Research Inc., Waltham, MA) according to the manufacturer’s instructions. Primers used for targets amplification were displayed as follows: TUG1: forward 5′-CTGAAGAAAGGC AACATC-3′; reverse: 5′-GTAGGCTACTACAGGATTG-3′; HMGB1: 5′-ACACCCAAATCTTGATCAAG-3′; reverse: 5′-AGGACAGCT TTCAAAATGTGT-3′; β-actin: forward: 5′-TGAGAGGGAAATCGTG CGTGAC-3′; reverse: 5′-AAAGAGGAAGGCTTGAAAGAG-3′. Each sample was examined in triplicate and analysed by the comparative threshold cycle (Ct) method.

Western blotting

Protein expression levels were analysed by Western blot. Briefly, the cells were harvested and lysed on ice for 30 min in buffer. After centrifugation, the concentrations of protein were determined and separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Then, protein was transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated into rabbit anti-HMGB1 (1:1000; Cell Signaling Technology, USA), TNF-α (1:1000; Cell Signaling Technology, USA), IFN-γ (1:1000; Cell Signaling Technology, USA), Bcl-2 (1:1000; Cell Signaling Technology, USA), Bax (1:1000; Cell Signaling Technology, USA), cleaved caspase-3 (1:1000; Cell Signaling Technology, USA) and mouse anti-GAPDH (1:1000; Cell Signaling Technology, USA) at 4°C overnight. Then, the membrane were then incubated with secondary peroxidase-conjugated antibodies for 2 h. Blots were treated with chemiluminescence reagents (Santa Cruz).
as per the manufacturer's instructions and analysed by ImageJ software.

**Statistical analysis**

All statistical analysis was carried out using the SPSS 18.0 software (SPSS, Inc., Chicago, IL). All values are expressed as the mean ± standard deviation from at least three repeated individual experiments for each group. The significant differences were analysed using Student’s t-test or χ² analysis for difference between two groups, and one-way ANOVA followed by Kruskal–Wallis H test for multiple comparison. Differences were considered significant when \( p < .05 \).

**Results**

**Knock down of TUG1 protected OGD/R-induced myocardial I/R injury**

As shown in Figure 1(a), the lncRNA TUG1 was significantly overexpression in OGD/R-induced myocardial HL-1 cells \( (p < .05) \). The level of TUG1 expression was significantly decreased in si-TUG1 transfected group and significantly increased in pc-TUG1 transfected group \( (p < .05) \), compared to the control group. In addition, the cell viability significantly decreased after OGD/R or TUG1 overexpression treatment compared to the control cells, which reversed by TUG1 knock down. These data indicated that suppression of TUG1 could prevent OGD/R-induced myocardial I/R injury.

**Knock down of TUG1 inhibited inflammation in OGD/R-induced myocardial HL-1 cells**

As shown in Figure 2, the expression of TNF-α and IFN-γ were significantly up-regulated after OGD/R treatment compared to the control group \( (p < .05) \), while inhibited expression in the TUG1 knock down group \( (p < .05) \). These suggested that overexpression of TUG1-induced inflammation in OGD/R-induced myocardial HL-1 cells.

**Knock down of TUG1 inhibited OGD/R-induced myocardial HL-1 cells apoptosis**

As shown in Figure 3(a), overexpression of TUG1 induced the OGD/R-induced myocardial HL-1 cells apoptosis, which could be inhibited after si-TUG1 transfected \( (p < .05) \). Furthermore, the apoptosis-related proteins detection showed that Bcl-2 and pro-caspase-3 were significantly decreased and Bax and cleaved caspase-3 were significantly increased in OGD/R-induced myocardial HL-1 cells \( (p < .05) \). In addition, the expression changes of these apoptosis-related proteins were significantly reversed after knock down of TUG1 \( (p < .05) \). Those suggested that suppression of TUG1 inhibited the apoptosis of OGD/R-induced myocardial HL-1 cells.
Knock down of TUG1 protected OGD/R-induced myocardial I/R injury by inhibiting HMGB1 expression

As shown in Figure 4(a), HMGB1 was significantly up-regulated in OGD/R-induced HL-1 cells compared to the control group \( (p < .05) \), while significantly inhibited after suppression of TUG1 \( (p < .05) \). To further determine whether knock down of TUG1 protected OGD/R-induced myocardial I/R injury through regulating HMGB1 expression. The HMGB1 inhibitor was used to treat OGD/R-induced myocardial HL-1 cells. The results showed that the effects of TUG1 regulated inflammatory factors expression in OGD/R-induced myocardial HL-1 cells were reversed by HMGB1 inhibitor \( (Figure 4(b), p < .05) \). In addition, the percentage of apoptosis cells were significantly reversed after HMGB1 inhibitor treatment \( (Figure 4(c), p < .05) \). Those suggested that knock down of TUG1 protected OGD/R-induced myocardial I/R injury by inhibiting HMGB1 expression.

Discussion

Myocardial muscle ischaemia reperfusion injury is a common pathophysiological change in clinical vascular surgery. All kinds of cardiovascular surgery, especially the treatment of myocardial infarction, may be accompanied by myocardial ischemia reperfusion [28–30]. I/R is a double-edged sword. On the one hand, it can solve the phenomenon of the
function of cardiomyocytes. On the other hand, it may lead to the occurrence of reperfusion injury, which called MIRI [31–34]. MIRI could cause changes in the cellular environment, such as oxidative stress, inflammatory response, and cellular Ca\(^{2+}\) overload [35]. Those could lead to cell apoptosis or necrosis, and myocardial dysfunction is inevitable [36]. In the present study, we explored function and underlying mechanism of lncRNA TUG1 in the cardiomyocyte ischaemia reperfusion injury. Our study determined that TUG1 expression was markedly overexpression in OGD/R-induced myocardial cell lines. Moreover, TUG1 knockdown inhibited inflammation, apoptosis in OGD/R-induced myocardial cell lines. Furthermore, our findings suggest that TUG1 knockdown protect OGD/R-induced myocardial I/R injury by inhibiting HMGB1 expression.

More and more evidence have suggested that lncRNAs could be novel biomarkers for diagnosis and treatment of various diseases [37–40]. Yu et al. reported that IncRNA MALAT1 may increase cardiomyocyte autophagy and myocardial injury during I/R by negatively regulating miR-204 expression [20]. Li et al. reported that suppression of KCNQ1OT1 may prevent myocardial I/R injury following acute myocardial infarction via regulating AdipoR1 and involving in p38 MAPK/NF-kB signal pathway [18]. Yu et al. reported that IncRNA UCA1 modulates cardiomyocyte apoptosis by targeting miR-143 in myocardial ischemia-reperfusion injury [41]. LncRNA TUG1 was overexpressed in several kinds of cancer tissues and could function as an oncogene or tumour suppressor in different cancers [42–46]. However, its function in myocardial injury during I/R has not investigated yet. In the present study, it is firstly reported the LncRNA TUG1 overexpression in OGD/R-induced myocardial cell lines and TUG1 knockdown protect OGD/R-induced myocardial I/R injury by inhibiting HMGB1 expression.

In the early stage of reperfusion, IRI can induce nuclear transfer of nuclear factor-κB and causes serum pro-inflammatory factors such as TNF-α, IL-6 increasing [47,48]. In the present study, HMBG1, TNF-α and IL-6 could regulate inflammation response and cell damage in the early stage of MIRI injury. Simultaneously, HMGB1 could induce TNF-α and IL-6 release and further aggravation of myocardial IRI [49]. Zhao et al. reported that down-regulation of nuclear HMGB1 reduces ischemia-induced HMGB1 release and protects against liver IRI, which is helpful for better understanding the role of HMGB1 in organ IRI [50]. Li et al. reported that miR-26a inhibited HMGB1 expression and attenuated cardiac ischaemia-reperfusion injury [51]. However, how it is regulated in myocardial ischaemia-reperfusion injury is not quite clear. In the present study, HMBG1 was significantly up-regulated in OGD/R-induced HL-1 cells. HMBG1 inhibitor could reverse the TUG1 regulated inflammatory factor expression, apoptosis. Our study presented a positive correlation between TUG1 and HMGB1 expression.

However, the molecule mechanism of lncRNA TUG1 inhibited HMGB1 expression in OGD/R-induced myocardial cells lines could not be well documented in the current study due to its limit of retrospective cohort. Additional in vivo experiments and clinical trials are warranted to justify this approach for lncRNA TUG1 target therapy in the future.

In conclusion, our findings determine that suppression of lncRNA TUG1 may prevent myocardial I/R injury following acute myocardial infarction via inhibiting HMGB1 expression. TUG1 may serve as a potential biomarker or therapeutic target for acute myocardial infarction. Further studies are still needed to verify our findings and hypothesis.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

**References**

[1] Chen G, Shen Y, Xu T, et al. Rapid detection of acute myocardial infarction-related miRNA based on a Capture-interCalation-electroCatalysis (3C) strategy. Biosens Bioelectr. 2016;77:1020–1025.
[2] Echouffo-Tcheugui JB, Kolte D, Khera S. Diabetes mellitus and cardiovascular shock complicating acute myocardial infarction. Am J Med. 2018;131:778–786.
[3] Takahashi M, Kondo Y, Senoo K, et al. Incidence and prognosis of cardiopulmonary arrest due to acute myocardial infarction in 85 consecutive patients. J Cardiol. 2018;72:343–349.
[4] Gao CK, Liu H, Cui CJ, et al. Roles of microRNA-195 in cardiomyocyte apoptosis induced by myocardial ischemia-reperfusion injury. J Genet. 2016;95:99–108.
[5] Feng L, Liu W, Yang J, et al. Effect of Hexadecyl Azelaoyl Phosphatidylcholine on cardiomyocyte apoptosis in myocardial ischemia-reperfusion injury: a hypothesis. Med Sci Monit. 2018;24:2661–2667.
[6] Feng S, Xiaoyuan L, Smith SC, et al. Acute myocardial infarction in patients with paraplegia: characteristics, management, and outcome. Am J Med. 2018;131:574.
[7] Chen L, Wang H, Gao F, et al. Functional genetic variants in the SIRT5 gene promoter in acute myocardial infarction. Gene. 2018;675:233–239.
[8] Kang R, Livesey KM, Zeh L, et al. HMGB1: a novel Beclin 1-binding protein active in active in autophagy. Autophagy. 2010;6:1209–1211.
[9] Xu Z, Jin Y, Yan H, et al. High-mobility group box 1 protein-mediated necroptosis contributes to dasatinib-induced cardiotoxicity. Toxicol Lett. 2018;296:39–47.
[10] Wang JS, Sheu WHH, Lee WJ, et al. Levels of serum high mobility group box 1 were independently associated with cardiovascular risk in patients undergoing coronary angiography. Clinical Chimica Acta. 2018;483:130–134.
[11] Mardente S, Mari E, Consorti F, et al. HMGB1 induces the overexpression of miR-222 and miR-221 and increases growth and motility in papillary thyroid cancer cells. Oncol Rep. 2012;28:2285–2289.
[12] Choey JY, Choi CH, Park KY, et al. High-mobility group box 1 protein mediates monosodium urate crystal-induced inflammation in human U937 macrophages. Biochem Biophys Res Commun. 2018;503:3248–3255.
[13] Song JH, Kim JY, Piao C, et al. Delivery of the high-mobility group box 1 box 1 box 1 peptide using heparin in the acute lung injury animal models. J Control Release. 2016;234:33–40.
[14] Yan W, Chang Y, Liang X, et al. High-mobility group box 1 activates caspase-1 and promotes hepatocellular carcinoma invasive- ness and metastases. Hepatology. 2012;55:1863–1875.
[15] Galllauria F, Cirillo P, D’agostino M, et al. Effects of exercise training on high-mobility group box-1 levels after acute myocardial infarction. J Cardiac Fail. 2011;17:108–114.
[16] Tong S, Zhang L, Joseph J, et al. Celastrol pretreatment attenuates rat myocardial ischemia/ reperfusion injury by inhibiting high mobility group box 1 protein expression via the P38Akt pathway. Biochem Biophys Res Commun. 2018;497:843–849.
Evonuk KS, Prabhu SD, Young ME, et al. Myocardial ischemia/reperfusion impairs neurogenesis and hippocampal-dependent learning and memory. Brain Behav Immun. 2017;61:266–273.

Agrawal V, Gupta JK, Qureshi SS, et al. Role of cardiac renin angiotensin system in ischemia reperfusion injury and preconditioning of heart. Indian Heart J. 2016;68:856–861.

Meng Z, Song MY, Li CF, et al. shRNA interference of NLRP3 inflammasome alleviates ischemia reperfusion-induced myocardial damage through autophagy activation. Biochem Pharmacol. 2017;94:728–735.

Yu L, Wang L, Li H, et al. Characterization of lncRNA expression profile and identification of novel lncRNA biomarkers to diagnose coronary artery disease. Atherosclerosis. 2018;275:359–367.

Shao M, Liu W, Wang Y. Differentially expressed lncRNAs as potential prognostic biomarkers for glioblastoma. Cancer Genet. 2018;226–227:23–29.

Zhou Q, Liu J, Quan J, et al. LncRNAs as potential molecular biomarkers for the clinicopathology and prognosis of glioma: A systematic review and meta-analysis. Gene. 2018;668:77–86.

Mishra S, Shah MI, Sarkar M, et al. Integrated analysis of non-coding RNAs for the identification of promising biomarkers in interstitial lung diseases. Gene. Rep. 2018;11:87–93.

Yu SY, Dong B, Zhou SH, Tang L. LncRNA UCA1 modulates cardiomyocyte apoptosis by targeting miR-143 in myocardial ischemia-reperfusion injury. Int J Cardiol. 2017;247:31.

Yu XH, Guo W, Zhang J, et al. Long non-coding RNA (lncRNA) TUG1, and the prognosis of cancer: a meta-analysis. Cell Mol Biol (Noisy-le-Grand). 2017;63:36–39.

Fan S, Yang Z, Ke Z, et al. Downregulation of the long non-coding RNA TUG1 is associated with cell proliferation, migration, and invasion in breast cancer. Biomed Pharmacother. 2017;95:1636–1643.

Li C, Gao Y, Li Y, et al. TUG1 mediates methotrexate resistance in colorectal cancer via miR-186/CPEB2 axis. Biochem Pharmacol. Commum. 2017;491:662–557.

Li S, Zhang S, Wang P, et al. LncRNA TUG1 regulates the oral squamous cell carcinoma progression possibly via interacting with Wnt/β-catenin signaling. Gene. 2017;608:49–57.

Ma F, Wang SH, Cai Q, et al. Long non-coding RNA TUG1 promotes cell proliferation and metastasis by negatively regulating miR-300 in gallbladder carcinoma. Biomed Pharmacother. 2017;88:863–869.

Ma F, Wang SH, Cai Q, et al. Long non-coding RNA TUG1 promotes cell proliferation and metastasis by negatively regulating miR-300 in gallbladder carcinoma. Biomed Pharmacother. 2017;88:863–869.

Fan S, Yang Z, Ke Z, et al. Downregulation of the long non-coding RNA TUG1 is associated with cell proliferation, migration, and invasion in breast cancer. Biomed Pharmacother. 2017;95:1636–1643.

Li C, Gao Y, Li Y, et al. TUG1 mediates methotrexate resistance in colorectal cancer via miR-186/CPEB2 axis. Biochem Pharmacol. Commum. 2017;491:662–557.

Li S, Zhang S, Wang P, et al. LncRNA TUG1 regulates the oral squamous cell carcinoma progression possibly via interacting with Wnt/β-catenin signaling. Gene. 2017;608:49–57.

Ma F, Wang SH, Cai Q, et al. Long non-coding RNA TUG1 promotes cell proliferation and metastasis by negatively regulating miR-300 in gallbladder carcinoma. Biomed Pharmacother. 2017;88:863–869.

Li C, Gao Y, Tian J, et al. Sophocarpine administration preserves myocardial function from ischemia-reperfusion in rats via NF-κB inactivation. J Ethnopharmacol. 2011;135:620–625.

Yamauchi K, Nakano Y, Imai T, et al. A novel nuclear factor erythroid 2-related factor 2 (Nrf2) activator SRF attenuates brain injury after ischemia reperfusion in mice. Neuroscience. 2016;333:302–310.

Hu X, Cui B, Zhou X, et al. Ethyl pyruvate reduces myocardial ischemia and reperfusion injury by inhibiting high mobility group box 1 protein in rats. Mol Biol Rep. 2012;39:227–231.

Zhao G, Fu C, Wang L, et al. Down-regulation of nuclear HMGB1 reduces ischemia-induced HMGB1 translocation and release and protects against liver ischemia-reperfusion injury. Sci Rep. 2017;7:1–11.

Li Y, Xin L, Wang X. MicroRNA 26a inhibits HMGB1 expression and attenuates cardiac ischemia-reperfusion injury. J Pharmacol Sci. 2016;131:6–12.