SFI Reduces the Nucleocytoplasmic Transportation of HMGB1 by Upregulating HDAC3 in LPS-induced RAW264.7 Cells

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ABSTRACT

Shenfu injection (SFI) is widely used for treating endotoxin shock in China. In the present study, to investigate the anti-inflammatory effects of SFI and further explored the potential mechanism of HMGB1 nuclear translocation, we established a vitro cell model provoked by lipopolysaccharide (LPS), observed nucleocytoplasmic translocation of high mobility group box 1 (HMGB1) and the relationship between histone deacetylase 3 (HDAC3) and HMGB1 under SFI intervention. The results showed that SFI upregulated the transcription and expression of HDAC3 in RAW264.7 cells, inhibited the nuclear to cytoplasmic translocation of HMGB1 and its subsequent extracellular release, and depressed the secretion of HMGB1, interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α). However, targeted knockdown of HDAC3 induced an increase in HMGB1 translocation to the cytoplasm, and HMGB1 localization was not altered significantly following LPS stimulation. SFI failed to reverse the abnormal localization of HMGB1. These results suggested that SFI may inhibit LPS-induced HMGB1 nuclear translocation in RAW264.7 cells through upregulating HDAC3 expression, thereby inhibiting its downstream pathway and suppressing inflammatory response.

Keywords: Cytoplasm; Prognosis; Synthesis

INTRODUCTION

Endotoxin shock is a highly lethal systemic inflammatory response syndrome. Inhibition of HMGB1 secretion can ameliorate inflammatory response and improve the prognosis [1]. HMGB1 is a non-histone DNA-binding protein located in nucleus, and whose synthesis, secretion and release are closely related to the extent of the inflammatory response [2-5]. During inflammation HMGB1 can be secreted actively by activated macrophages, dendritic cells or endothelial cells, or released passively by necrotic cells [6]. Extracellular HMGB1 functions as a damage-associated molecular pattern molecule and triggers the inflammation cascade inducing the secondary damage of multiple diseases, which is the key inflammatory mediator of mortality in endotoxin shock [7-9]. Meanwhile, in view of the fact that the process of nucleocytoplasmic translocation and secretion of HMGB1 is characterized by late occurrence and long duration, it is considered that inhibiting the secretion of HMGB1 may be an effective strategy for the treatment of endotoxin shock and expanding the therapeutic time window, which has important research value [10-12].

Depending on the theory of traditional Chinese medicine (TCM) syndrome differentiation, endotoxin shock belongs to the category of "prostration syndromes" in TCM. Clinically, SFI has been used to treat endotoxin shock in China and has good clinical efficacy [13-15]. Lysine acetylation, a dynamic and reversible protein post-translational modification, is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs) [16,17]. The hyper-acetylation of HMGB1 catalyzed by HATs is an important condition leading to the extranuclear migration of HMGB1, whereas HDACs repressing it [18-20]. Upregulation of HDACs expression can reduce inflammatory injury by inhibiting HMGB1 release [21,22]. Therefore, maintaining an equilibrium between HATs and HDACs may be an effective way to treat inflammatory diseases by inhibiting the nuclear translocation of HMGB1.

Previous studies have shown that SFI can effectively inhibit LPS-induced HMGB1 release in macrophages and the inflammatory response in rat models of endotoxin shock [23]. Moreover, HDAC3 expression was significantly increased by hypaconitine which is the main component of SFI [24]. HDAC3 belongs to the class I HDACs and is widely expressed [25]. However, whether the upregulation of HDAC3 expression by SFI is the key factor for SFI to inhibit HMGB1 nuclear translocation remains to be further studies. In this study, LPS-stimulated RAW264.7 cells were used as the inflammatory cell model. To explore the anti-inflammatory mechanism of SFI inhibiting HMGB1 nuclear translocation from the perspective of acetylation modification of HMGB1.

MATERIALS AND METHODS

Materials

Shenfu injection (cat. no. Z51020664) was purchased from Ya'an Sanjiu Pharmaceutical (Sichuan, China). LPS (cat. no. 039M4004V) was purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Cell culture and drug treatment

The murine macrophage cell line RAW264.7 (Shanghai Institute of Cell Biology, Shanghai, China) was grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM; Gibco, USA) with 10% fetal bovine serum (FBS; Gibco, USA).

After reaching 70~80% confluence, the RAW264.7 cells were changed to serum-free medium. Cells in control group were not treated with any drugs, the LPS group was stimulated with 0.2 µg/mL LPS for 24 h, and cells in SFI group were co-treated with SFI (3, 6 or 12 µL/mL) plus LPS (0.2 µg/mL) for 24 h. Each group had 3 replicate wells and was assayed in triplicate.

Enzyme-linked immunosorbent assay

The cell supernatant was collected and the levels of HMGB1, TNF-α, and IL-1β were measured with the mouse HMGB1 ELISA kit, the mouse TNF-α ELISA kit and the mouse IL-1β ELISA kit (cat. no. M173318-215a, M170318102a, M190325-318a; Neobioscience Technology Co., Ltd.; Shenzhen, China) following the manufacturers' instructions.

Immunofluorescence assay

After various treatments, the RAW264.7 cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.5% Triton X-100 in PBS, blocked with 10% goat serum for 1 h, incubated with anti-HMGB1 (cat. no. 2600-1; dilution, 1:200; Epitomics; Abcam, Cambridge, MA, USA) antibody overnight at 4°C, incubated with the second antibody which is cy3-labeled goat anti-rabbit IgG (cat. no. P0183-1; dilution, 1:100; Beyotime; Shanghai, China) antibody for 1 h, counterstained with DAPI for 5 min, and then observed by fluorescence microscopy (Olympus Corporation, Tokyo, Japan).

RT-qPCR analysis

Total RNA was isolated from experimental RAW264.7 cells using TRIzol kit (cat. no. DP424; TIANGEN; Beijing, China) and cDNA was synthesized by using the reverse transcription kits (cat. no. DBI-2220; Shanghai, China). RT-qPCR amplification reactions were performed using 2 µL of cDNA as a template and the all-in-One qPCR master mix (AOPR-1200; GeneCopoeia; Rockville, MD, USA). The primer sequences used for RT-qPCR were constructed by Shanghai GenePharma Co., Ltd. (Shanghai, China) as shown in Table 1. After an initial incubation (95°C, 10 min), each qPCR protocol involves 40 cycles of denaturation (95°C, 10 sec), annealing (55°C, 20 sec) and elongation (72°C, 35 sec). Each sample was tested in triplicate and all relative gene expression values were normalized to β-actin using the $2^{-\Delta\Delta Ct}$ method [26].

Western blot analysis

Proteins were extracted from RAW264.7 cells and fractioned

Table 1: Primer list for RT-qPCR.

Gene name	Primer sequences (5'-3')	Product (bp)
β-actin	F: GAGGTATCCTGACCCTGAAGTA	104
	R: CACACGCAGCTCATTGTAGA	
TNF-α	F: CTACCTTGTTGCCTCCTCTTT	116
	R: GAGCAGAGGTTCAGTGATGTAG	
HMGB1	F: CTGATTGGCTGGAGGAATGT	99
	R: ATACCACCAAGTTTCCCATCTC	
IL-1β	F: GCAGCATCTCGACAAGAGCTT	91
	R: GCTCCACGGGCAAGACATAG	
HDAC3	F: ATGCCTTCAACGTGGGTGAC	99
	R: TTAGCTGTGTTGCCCCTTGC	

by 12% SDS-PAGE and blotted onto a PVDF membrane (Millipore). Then the PVDF membranes were blocked with 5% fat-free milk at room temperature for 1 h. Subsequently, the membranes were incubated by monoclonal anti-HMGB1 (cat. no. 2600-1; dilution, 1:1000; Abcam), anti-HDAC3 (cat. no. S1306; dilution, 1:1000; Abcam), anti-GAPDH (cat. no. ab9485; dilution, 1:1000; Abcam) antibody overnight at 4˚C, followed by incubation with horse radish peroxidase (HRP)-labeled goat anti-rabbit IgG secondary antibody (cat. no. BA1054; dilution, 1:1000; Wuhan Boster Biological Technology, Ltd.; Wuhan, China). Every experiment was conducted three times, and the data were quantified using ImageJ software.

siRNA interference

SiRNA-1, siRNA-2, and siRNA-3 of HDAC3 were constructed by Borui Biotechnology Co., Ltd (Guangzhou, China). The siRNA sequences for HDAC3 were 5'-AUUGGUAUC-CUGGAGCUGCTTGCAGCUCCAGGAUACCAAUTT-3' (siRNA-1) and 5'-AAUGCCUUCAACGUGGGUGTTCAC-CCACGUUGAAGGCAUUTT-3' (siRNA-2) and 5'-UGUG-CCCUUACGAGAUGGCTTGCCAUCUCGUAAGGG-CACATT-3' (siRNA-3). Cells were transfected at 30-40% confluence with siRNA using Lipofectamine™2000 (cat. no. 52887; Invitrogen; Thermo Fisher Scientific, Inc.). The highest silencing efficiency pair of siRNA (HDAC3-siRNA-3) was selected for further experiments through western blot assays after 36 h.

RAW264.7 cells (5×10^3) were plated on glass coverslips in 24-well plates. Cells were divided into the control (CON), negative control siRNA (NC), HDAC3-siRNA, HDAC3-siRNA+LPS and HDAC3-siRNA+LPS+SFI 6 μL/mL group. Cells were transfected with siRNA-3 as described above. There were 3 wells in each group with 3 repeats. After incubation for 12 h, cells were treated with SFI and LPS for 24h. After

HDAC3 knockdown, subcellular localization of HMGB1 from LPS-stimulated RAW264.7 cells was observed under the fluorescent microscope.

Statistical analysis

All the above experiments were carried out three times at least. All statistical analyses were conducted using SPSS 22.0 software (SPSS version 22.0, IBM Corp., USA). Results were transferred to GraphPad Prism 7 Software (GraphPad Software Inc., USA) for plotting graphics. The data obeying a normal distribution and had a homogeneity of variance were presented as x±s, using one-way ANOVA of variance for comparison between groups. Data were transformed when the variance was not homogeneous or when the data did not normally distributed, followed by one-way ANOVA. Value ($P<0.05$) was treated as statistically significant.

RESULTS

SFI can suppress the pro-inflammatory cytokines transcription and secretion in LPS-induced RAW264.7 cells.

To measure the levels of inflammatory factors, the RAW264.7 cells treated with SFI and LPS were analyzed by ELISA and RT-qPCR. (Figure 1 and 2). ELISA results presented in Figure 1. Compared with the control group, the secretion of HMGB1, IL-1β and TNF-α was significantly increased by LPS stimulation. Compared with the LPS group, SFI treatment resulted in a significant inhibition in HMGB1, IL-1β and TNF-α level. The results of RT-qPCR (Figure 2) were consistent with the findings of ELISA. We found SFI upregulated the mRNA level of HMGB1 (Figure 2b) and dose-dependently upregulated the level of IL-1β (Figure 2c) and TNF-α (Figure 2d) mRNA in LPS-stimulated RAW264.7 cells. These results indicated that SFI could sharply reduce the level of proinflammatory cytokines, and effectively inhibit the inflammatory response of LPS-stimulated RAW264.7 cells.
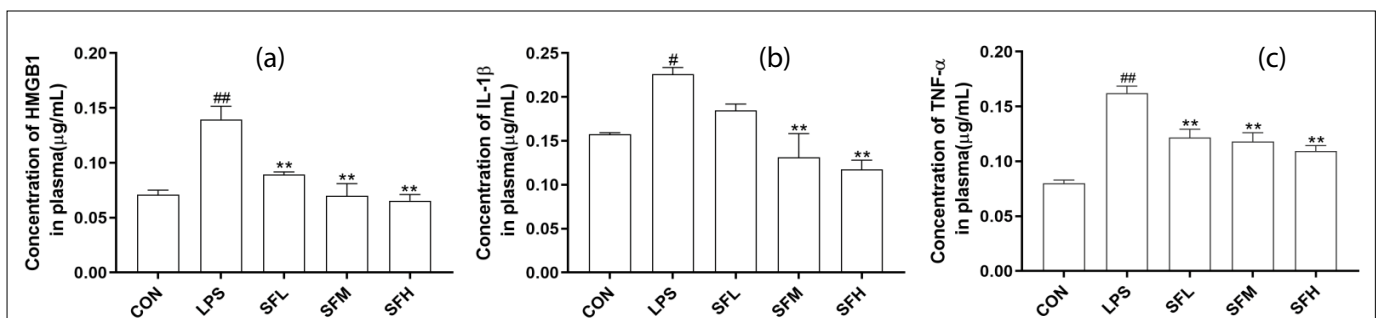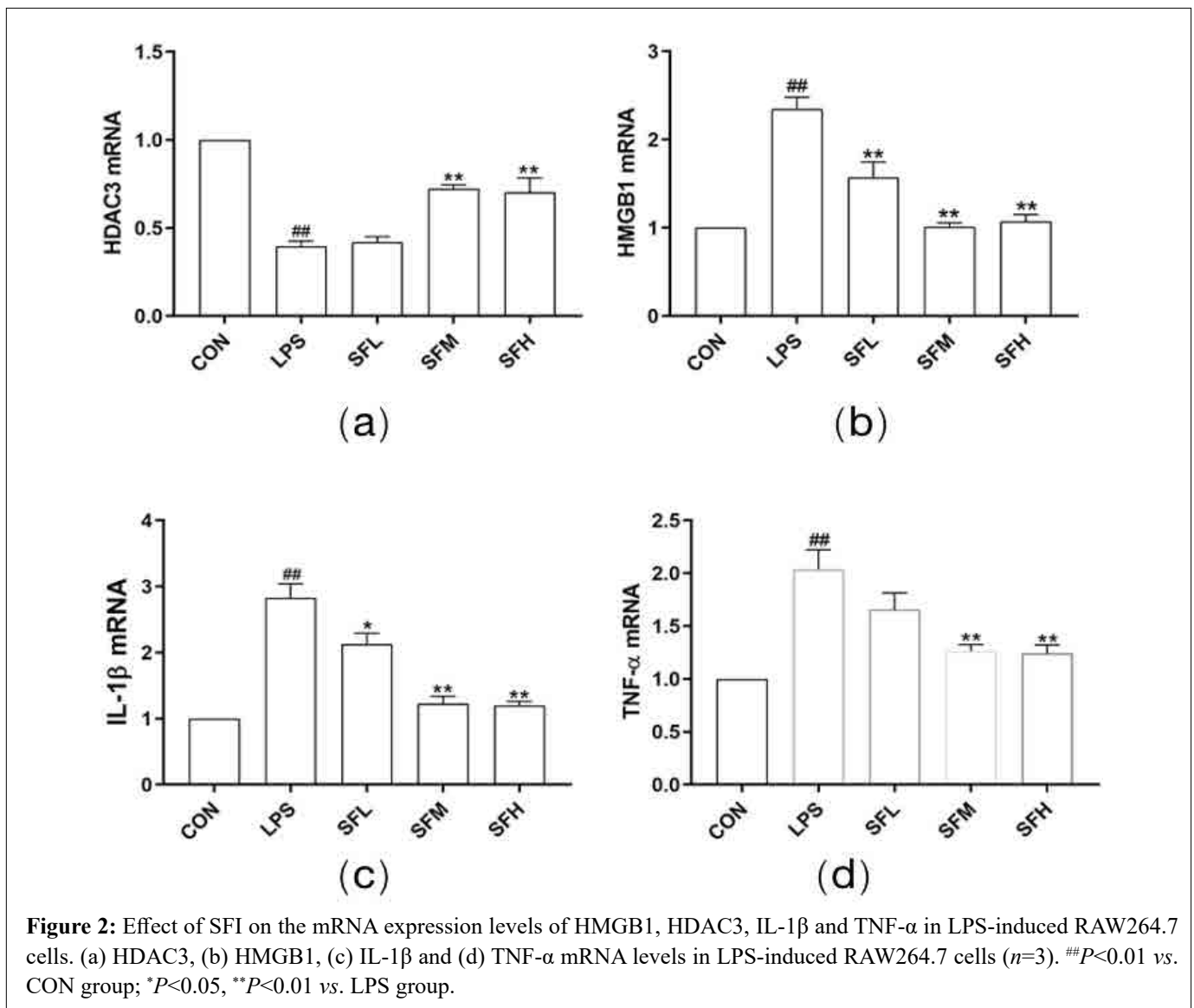

Figure 1: Effect of SFI on the levels of inflammatory factors in the supernatant of LPS-induced RAW264.7 cells in μg/mL. (a) HMGB1, (b) IL-1β and (c)TNF-α in the plasma of LPS-induced RAW264.7 cells (*n*=3). #*P*<0.05 and ##*P*<0.01 *vs*. CON group; **P*<0.01 *vs*. LPS group.

Figure 2: Effect of SFI on the mRNA expression levels of HMGB1, HDAC3, IL-1β and TNF-α in LPS-induced RAW264.7 cells. (a) HDAC3, (b) HMGB1, (c) IL-1β and (d) TNF-α mRNA levels in LPS-induced RAW264.7 cells (n=3). $^{##}P<0.01$ *vs*. CON group; $^{*}P<0.05$, $^{**}P<0.01$ *vs*. LPS group.
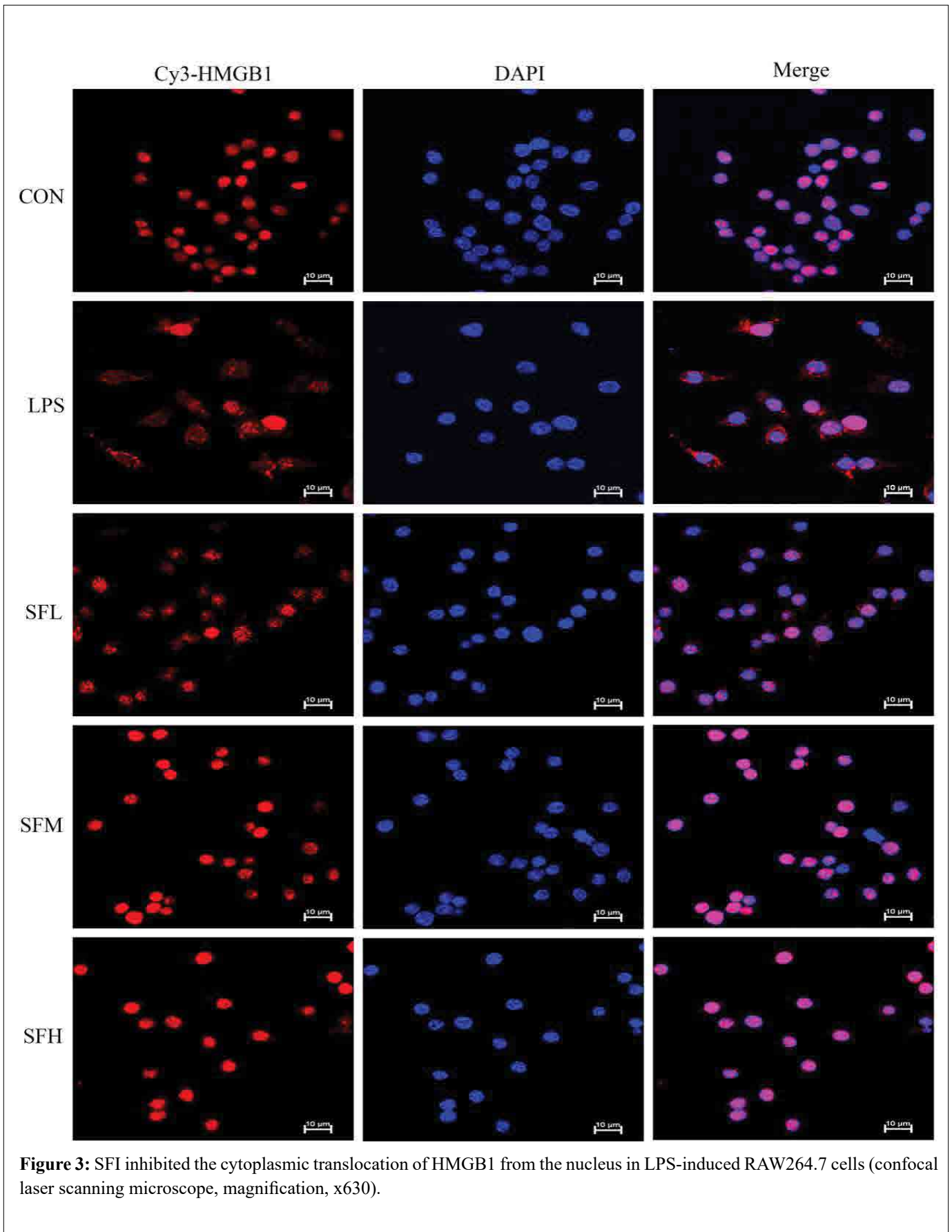
SFI inhibited LPS-treated HMGB1 translocation from nucleus to cytoplasmic compartment
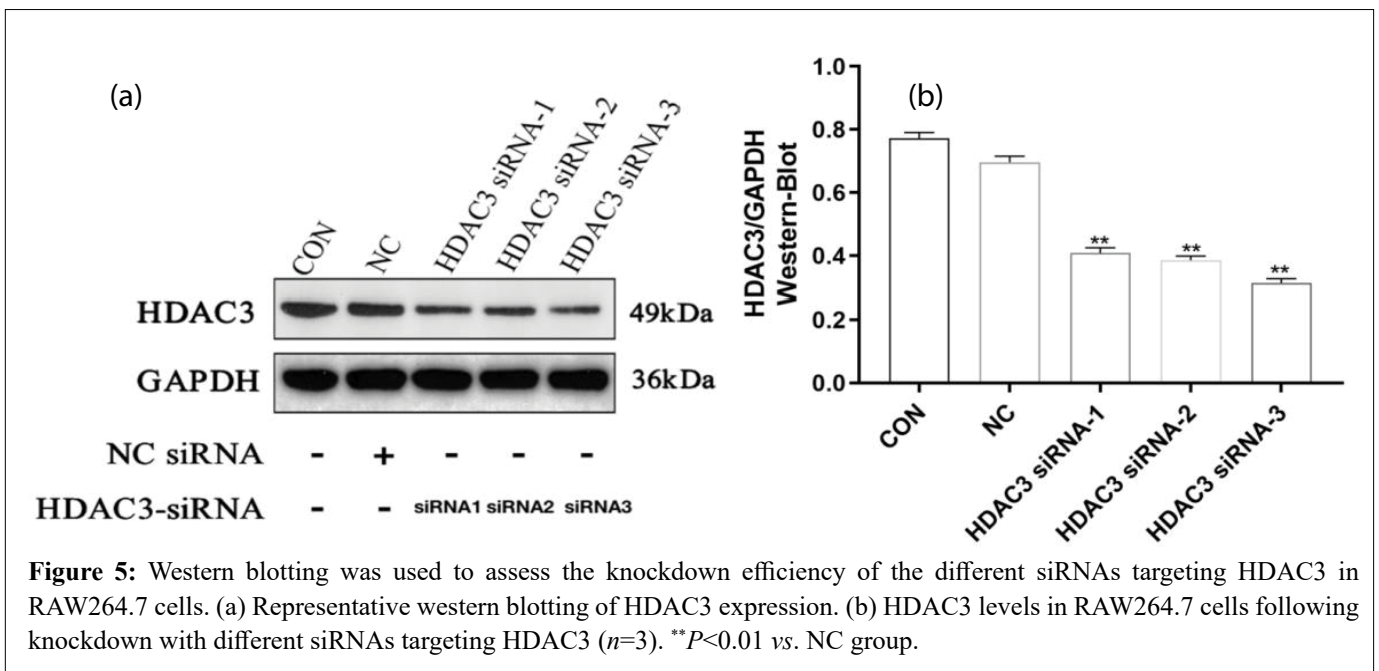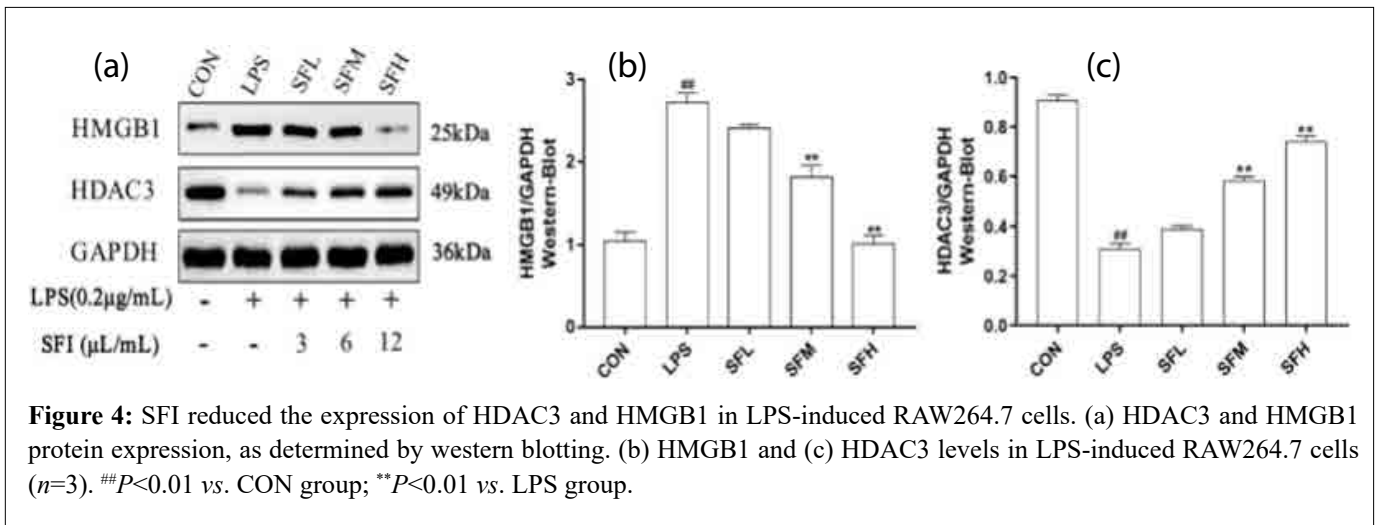
Subcellular localization of HMGB1 was observed by immunofluorescence (Figure 3). HMGB1 was stained with Cy3 (red), while nuclei were counterstained with DAPI (blue). In the control group, red fluorescence almost overlapped with blue fluorescence, this also suggests that HMGB1 was predominantly concentrated in the nuclei under normal conditions. In the LPS group, HMGB1 was mainly concentrated in the cytoplasm, indicating that intranuclear HMGB1 can translocate to the cytoplasm under LPS induction. Furthermore, some red granules and cell membranes into close proximity, suggesting that HMGB1 might be released into the extracellular space. After SFI treatment, LPS-induced HMGB1 nucleocytoplasmic

translocation was blocked and similar to the control group. SFI enhanced the nuclear localization of HMGB1 and at the same time reduced cytoplasmic HMGB1. Our results suggested that SFI prevented the HMGB1 cytoplasmic relocation in LPS-induced RAW264.7 cells.

SFI upregulated the mRNA and protein expression level of HDAC3 in RAW264.7 cells

As is well known, acetylation, nucleocytoplasmic translocation and secretion of HMGB1 play vital roles in inflammatory response, which is regulated by its upstream factor HDACs. To investigate whether the process of SFI inhibits HMGB1 nuclear translocation triggered acetylation of HMGB1, we examined HMGB1 and HDAC3 content by western blotting (Figure 4) and the mRNA expression of HMGB1 and HDAC3 (Figure 2b and a) by PCR in RAW264.7 cells following

Figure 3: SFI inhibited the cytoplasmic translocation of HMGB1 from the nucleus in LPS-induced RAW264.7 cells (confocal laser scanning microscope, magnification, x630).

Figure 4: SFI reduced the expression of HDAC3 and HMGB1 in LPS-induced RAW264.7 cells. (a) HDAC3 and HMGB1 protein expression, as determined by western blotting. (b) HMGB1 and (c) HDAC3 levels in LPS-induced RAW264.7 cells (*n*=3). $^{##}P<0.01$ *vs.* CON group; $^{**}P<0.01$ *vs.* LPS group.

Figure 5: Western blotting was used to assess the knockdown efficiency of the different siRNAs targeting HDAC3 in RAW264.7 cells. (a) Representative western blotting of HDAC3 expression. (b) HDAC3 levels in RAW264.7 cells following knockdown with different siRNAs targeting HDAC3 (*n*=3). $^{**}P<0.01$ *vs.* NC group.
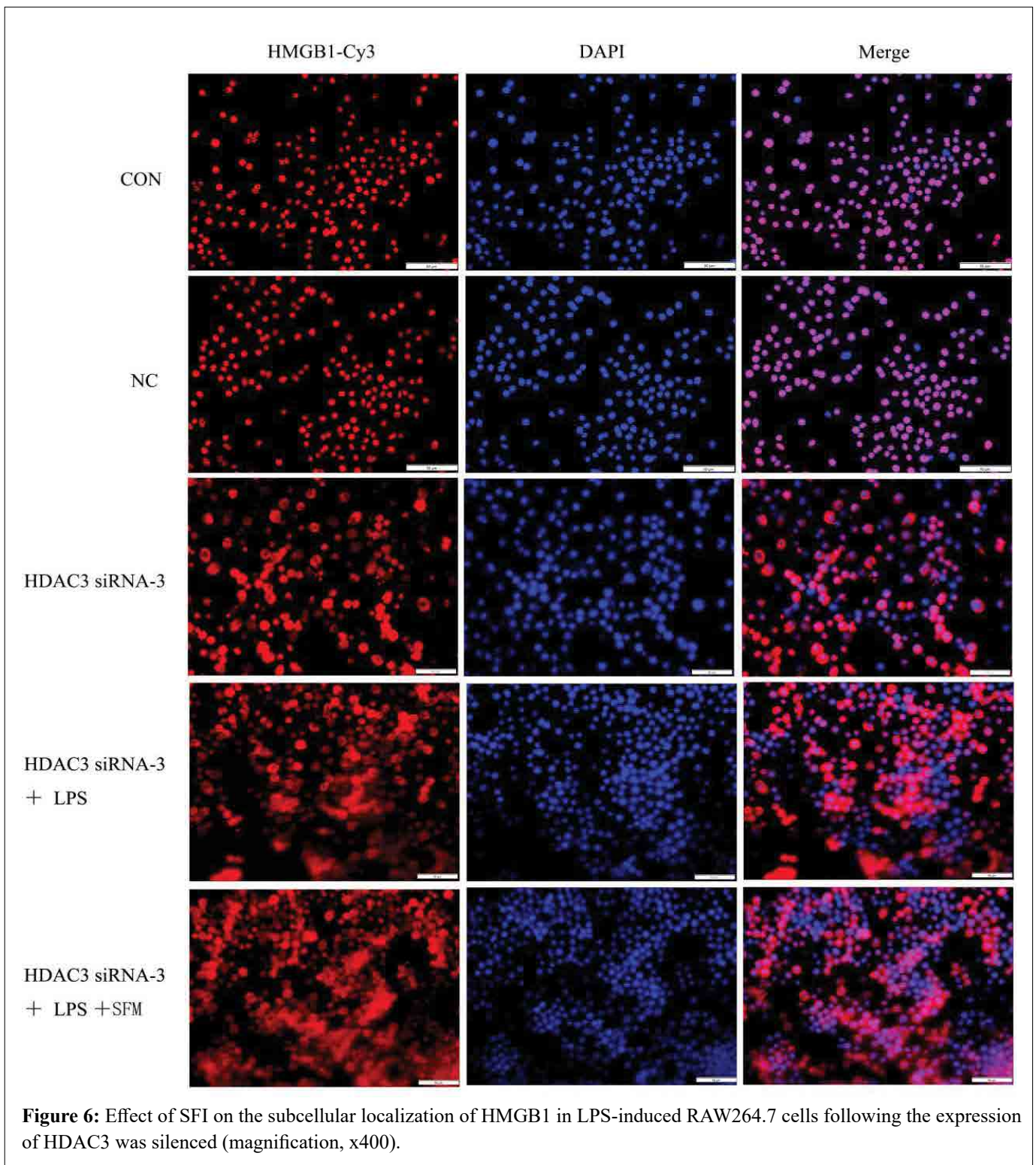
stimulation with SFI and LPS. Compared with the control group, the level of HMGB1 was significantly increased in both mRNA and protein levels by LPS stimulation, while the level of HDAC3 suppressed. Compared with the LPS group, SFI dose-dependently upregulated the mRNA and protein expression level of HDAC3 and the level of HMGB1 was significantly reduced. The above results suggested that SFI may reverse LPS-induced HMGB1 acetylation and nuclear-cytosolic translocation by enhancing the expression of HDAC3.

SFI had no effect on the relocation of HMGB1 after HDAC3 knockdown.

To further detect whether the involvement of SFI in the relocation of HMGB1 associates with HDAC3. First, the siRNA sequences targeting HDAC3 applied in this study were selected from 3 pairs of siRNA sequences (siRNA-1, siRNA-2, and siRNA-3) by western blotting. We selected the siRNA sequences (siRNA-3) with the best suppressive effects for subsequent studies (Figure 5). Thereafter, RAW264.7 cells were transfected with siRNA-3. Next, subcellular localization of HMGB1 in the cells with LPS and SFI treatments examined by immunofluorescence staining (Figure 6). Combined with results of the previous experiment, the effect of SFI at middle dose was equivalent in treatment performance to the high dose. And considering the toxicity of SFI, the middle dose of SFI was chosen for interference assays.

The results are shown in the figure. A large number of HMGB1 appeared in the cytoplasm in the HDAC3 siRNA-3 group compared with the CON group; Compared with the HDAC3

Figure 6: Effect of SFI on the subcellular localization of HMGB1 in LPS-induced RAW264.7 cells following the expression of HDAC3 was silenced (magnification, x400).

siRNA-3 group, the localization of HMGB1 did not change significantly following stimulation with LPS; Compared with the HDAC3 siRNA-3+LPS group, the subcellular localization of HMGB1 has no obvious change in the HDAC3 siRNA-3+LPS+SFM group. We observed that SFI prevented HMGB1 nucleocytoplasmic translocation in LPS-induced RAW264.7 cells, and silencing of HDAC3 suppressed the effect of SFI, suggesting an vital role for HDAC3 in SFI-induced effects. The above results confirmed the regulatory effect of HDAC3 on nuclear localization of HMGB1, and further verify that SFI

can inhibit HMGB1 extranuclear migration by upregulating the expression of HDAC3.

DISCUSSION

Inflammatory is the initial response of immune system to infection or injury. Although mmune response can clear most infections, but unrestrained immune responses can lead to many diseases including endotoxin shock. The prolonged or exaggerated immune responses are associated with a cytokine storm [27]. Extracellular HMGB1 secretion triggers the amplification of the inflammatory cascade, which may be a late key mediator of endotoxin lethality. Compared with early pro-inflammatory factors, HMGB1 appeared later and lasted longer that it participated in most pathophysiological processes of endotoxin shock [11,28]. The delayed kinetics of HMGB1 release is correlated with the severity of the disease and subsequent fatal outcome, and there is a long therapeutic time window for treating these inflammatory diseases with HMGB1 as the target.

HMGB1 translocation is related to the hyper-acetylation of HMGB1, inhibiting the acetylation levels of HMGB1 may be an effective way to inhibit the translocation of HMGB1 to extranuclear. HMGB1 nuclear translocation of HMGB1 is thought to hyper-acetylation of two nuclear localization sequences (27-29 and 181-183 lysine sites) when cells were primed with noxious stimuli. Some studies confirmed that HATs/HDACs can modify the lysine sites of non-histones, and increase the expression of HATs (such as P300/CBP) and/or decrease the expression of HDACs (such as HDAC3), resulting in increasing acetylation and translocation of HMGB1, indicating that HATs/HDACs play pivotal roles in modulating HMGB1 nuclear translocation and secretion [29,30].

SFI is commonly used in the clinical treatment for endotoxin shock. However, the detailed anti-inflammatory mechanism of SFI requires further investigation and improvement [31]. Ginsenosides and aconitum alkaloids, the main active components of SFI, which can ameliorate inflammation response and microcirculatory disturbance [14,32-34]. In one, hypoaconitine can promote the transcription and expression of HDAC3 gene in endothelial cells [24], but we currently have little mechanistic understanding of its effect on HDAC3 of macrophages. Combined with the previous research by our group showed SFI can inhibit HMGB1/Toll-like receptor 4 (TLR4) and HMGB1/nuclear transcription factor kappa B (NF-κB) signal pathway, which inhibits the exnuclear and secretion of HMGB1, educes inflammatory reaction and cell damage [23]. Therefore, our group hypothesized that HDAC3-HMGB1 pathway is the key to

SFI regulated HMGB1 nuclear translocation and exerted anti-inflammatory effects.

Based on those, in this study, RAW264.7 cells were stimulated with LPS for 24 h to establish the inflammation model. Meanwhile, the results of ELISA and PCR also demonstrated that the expression and secretion of pro-inflammatory factors HMGB1, TNF-α and IL-1β were significantly increased at the 24-hour time point, and immunofluorescence revealed that LPS resulted in translocation of HMGB1, commonly located in the nucleus, to extranuclear locations, indicating that the inflammatory cascade was activated, at the same time, HDAC3 was significantly upregulated at both mRNA and protein levels. Following SFI intervention, this condition was significantly reversed, LPS enhanced HMGB1 expression and translocation were inhibited, and HDAC3 expression was also reversed during the inflammatory response.It can be seen that SFI reduced the possibility of HMGB1 as a late factor of lethal inflammation from the source. Next, cells were treated with HDAC3 gene silencing technology, LPS and SFI. The localization of HMGB1 was observed by immunofluorescence method, and the results showed that when HDAC3 gene was silenced, SFI could not inhibit HMGB1 the extranuclear migration of in LPS-induced RAW264.7 cells.

This study found that SFI has an effect on the expression of HDAC3 in LPS-induced macrophages, and may block HMGB1 nucleocytoplasmic migration by increasing HDAC3.This study provides a new research direction and target for SFI in epigenetic treatment of endotoxin shock and enriches the anti-inflammatory mechanism of traditional Chinese medicine. HMGB1 is a late inflammatory factor and its secretion lasts for a long time, and studying the upstream target of SFI regulating HMGB1nucleocytoplasmic translocation and treating acute inflammation through this target can expand the therapeutic time window. However, this study only selected the time point of 24 h to study the mechanism of SFI inhibiting HMGB1 extranuclear migration, so the specific inhibition duration remains to be studied.

CONCLUSIONS

Extracellular HMGB1 is a pro-inflammatory factor, and inhibiting HMGB1 extranuclear migration is an effective way to treat inflammation. The nucleocytoplasmic translocation of HMGB1 is reversible. When the activity of cell deacetylase increase, the deacetylated HMGB1 in cytoplasm can return to the nucleus.

In conclusion, in this study, SFI upregulated the expression of HDAC3 , which may be an important mechanism to inhibit HMGB1 acetylation and extranuclear migration in LPS-induced RAW264.7 cells.

REFERENCES

1. Deng C, Zhao L, Yang Z, Shang JJ, Wang CY, et al. (2022) Targeting HMGB1 for the treatment of sepsis and sepsis-induced organ injury. Acta Pharmacol Sin. 43: 520-528.

2. Hou H F, Liu Y, Zhang X, Han Z, Chen T (2022) The value of postoperative HLA-DR expression and high mobility group box 1 level in predictive diagnosis of sepsis in percutaneous nephrolithotomy surgery. Ren Fail 44: 1338-1344.

3. Karakike E, Adami M E, Lada M, Gkavogianni T, Koutelidakis IM, et al. (2019) Late Peaks of HMGB1 and Sepsis Outcome: Evidence for Synergy With Chronic Inflammatory Disorders. Shock 52: 334-339.

4. Yoo H, Im Y, Ko RE, Lee JY, Park J, et al. (2021) Association of plasma level of high-mobility group box-1 with necroptosis and sepsis outcomes. Sci Rep 11: 9512.

5. Gibot S, Massin F, Cravoisy A, Barraud D, Nace L, et al. (2007) High-mobility group box 1 protein plasma concentrations during septic shock. Intensive Care Med 33: 1347-1353.

6. Venereau E, De Leo F, Mezzapelle R, Carrecia G, Musco G, et al. (2016) HMGB1 as biomarker and drug target. Pharmacol Res 111: 534-544.

7. Vijayakumar EC, Bhatt LK, Prabhavalkar KS (2019) High Mobility Group Box-1 (HMGB1): A Potential Target in Therapeutics. Curr Drug Targets 20: 1474-1485.

8. Wang H, Bloom O, Zhang M, Ombrellino M, Che J, et al. (1999) HMG-1 as a late mediator of endotoxin lethality in mice. Science 285: 248-251.

9. Deng M, Tang Y, Li W, Wang X, Zhang R, et al. (2018) The Endotoxin Delivery Protein HMGB1 Mediates Caspase-11-Dependent Lethality in Sepsis. Immunity 49: 740-753. e7.

10. Andersson U, Tracey K J (2003) HMGB1 in sepsis. Scand J Infect Dis 35: 577-584.

11. Chen D, Bellussi L M, Passali D, Chen L (2013) LPS may enhance expression and release of HMGB1 in human nasal epithelial cells *in vitro*. Acta Otorhinolaryngol Ital 33: 398-404.

12. Grégoire M, Tadié JM, Uhel F, Gacoun A, Piau C, et al. (2017) Frontline Science: HMGB1 induces neutrophil dysfunction in experimental sepsis and in patients who survive septic shock. J Leukoc Biol 101: 1281-1287.

13. Varon A, Varon J (2015) Shenfu, traditional Chinese medicine in sepsis: are we ready for it?. Am J Emerg Med 33: 1244-1245.

14. Ge A H, Li J, Donnapee S, et al. (2015) Simultaneous determination of 2 aconitum alkaloids and 12 ginsenosides in Shenfu injection by ultraperformance liquid chromatography coupled with a photodiode array detector with few markers to determine multicomponents. J Food Drug Anal 23: 267-278.

15. Xu C, Xia Y, Jia Z, et al. (2022) The curative effect of Shenfu-injection in the treatment of burn sepsis and its effect on the patient's immune function, HMGB, and vWF. Am J Transl Res 14: 2428-2435.

16. Von Knethen A, Brüne B (2019) Histone Deacetylation Inhibitors as Therapy Concept in Sepsis. Int J Mol Sci 20.

17. Narita T, Weinert B T, Choudhary C (2019) Functions and mechanisms of non-histone protein acetylation. Nat Rev Mol Cell Biol 20: 156-174.

18. Liu P, Zhao L, Loos F, et al. (2018) Epigenetic anticancer agents cause HMGB1 release *in vivo*. Oncoimmunology 7: e1431090.

19. Xu Q, Liu X, Mei L, et al. (2018) Paeonol Reduces the Nucleocytoplasmic Transportation of HMGB1 by Upregulating HDAC3 in LPS-Induced RAW264.7 Cells. Inflammation 41: 1536-1545.

20. Banerjee S, Rakshit T, Sett S, et al. (2015) Interactions of Histone Acetyltransferase p300 with the Nuclear Proteins Histone and HMGB1, As Revealed by Single Molecule Atomic Force Spectroscopy. J Phys Chem B 119: 13278-13287.

21. Wei S, Gao Y, Dai X, et al. (2019) SIRT1-mediated HMGB1 deacetylation suppresses sepsis-associated acute kidney injury. Am J Physiol Renal Physiol 316: F20-f31.

22. Chen X, Chen C, Fan S, et al. (2018) Omega-3 polyunsaturated fatty acid attenuates the inflammatory response by modulating microglia polarization through SIRT1-mediated deacetylation of the HMGB1/NF-κB pathway following experimental traumatic brain injury. J Neuroinflammation 15: 116.

23. Liu X, Ai F, Li H, et al. (2019) Anti-Inflammatory Effects of Shenfu Injection against Acute Lung Injury through Inhibiting HMGB1-NF-κB Pathway in a Rat Model of Endotoxin Shock[J]. Evid Based Complement Alternat Med 2019: 9857683.

24. Bai Y, Du S, Li F, et al. (2017) Histone deacetylase-high mobility group box-1 pathway targeted by hypaconitine suppresses the apoptosis of endothelial cells. Exp Biol Med (Maywood) 242: 527-535.

25. Singh AK, Bishayee A, Pandey AK (2018) Targeting Histone Deacetylases with Natural and Synthetic Agents: An Emerging Anticancer Strategy. Nutrients 10.

26. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402-408.

27. Fajgenbaum DC, June CH (2020) Cytokine Storm. N Engl J Med 383: 2255-2273.

28. Qu L, Chen C, Chen Y, Li Y, Tang F, et al. (2019) High-Mobility Group Box 1 (HMGB1) and Autophagy in Acute Lung Injury (ALI): A Review. Med Sci Monit 25: 1828-1837.

29. Volchuk A, Ye A, Chi L, Steinberg BE, Golednberg NM (2020) Indirect regulation of HMGB1 release by gasdermin D. Nat Commun 11: 4561.

30. Bonaldi T, Talamo F, Scaffidi P, Ferrera D, Porto A, et al. (2003) Monocytic cells hyperacetylate chromatin protein HMGB1 to redirect it towards secretion. Embo j 22: 5551-5560.

31. Wu J, Li Z, Yuan W, Zhang Q, Liang Y, et al. (2021) Shenfu injection improves cerebral microcirculation and reduces brain injury in a porcine model of hemorrhagic shock. Clin Hemorheol Microcirc 78: 175-185.

32. Yi YS (2021) New mechanisms of ginseng saponin-mediated anti-inflammatory action via targeting canonical inflammasome signaling pathways. J Ethnopharmacol 278: 114292.

33. Li J, Huang Q, Yao Y, Ji P, Mingyao E, et al. (2022) Biotransformation, Pharmacokinetics, and Pharmacological Activities of Ginsenoside Rd Against Multiple Diseases. Front Pharmacol 13: 909363.

34. Gu W, Li C, Yin W, Guo Z, Hou X, et al. (2012) Shen-fu injection reduces postresuscitation myocardial dysfunction in a porcine model of cardiac arrest by modulating apoptosis. Shock 38: 301-306.