Inhibition of hypoxia inducible factor-1α ameliorates lung injury induced by trauma and hemorrhagic shock in rats

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Aim: Ischemia/reperfusion is an initial triggering event that leads to gut-induced acute lung injury (ALI). In this study, we investigated whether hypoxia inducible factor-1α (HIF-1α) played a role in the pathogenesis of lung injury induced by trauma and hemorrhagic shock (T/HS).

Methods: Male Wistar rats underwent laparotomy and hemorrhagic shock for 60 min. Sham-shock animals underwent laparotomy but without hemorrhagic shock. After resuscitation for 3 h, the rats were sacrificed. Morphologic changes of the lungs and intestines were examined. Bronchoalveolar lavage fluid (BALF) was collected. Lung water content, pulmonary myeloperoxidase (MPO) activity and the levels of malondialdehyde (MDA), nitrite/nitrate, TNF-α, IL-1β, and IL-6 in the lungs were measured. The gene expression of pulmonary HIF-1α and iNOS, and HIF-1α transcriptional activity in the lungs were also assessed. The apoptosis in the lungs was determined using TUNEL assay and cleaved caspase-3 expression.

Results: Lung and intestinal injuries induced by T/HS were characterized by histological damages and a significant increase in lung water content. Compared to the sham-shock group, the BALF cell counts, the pulmonary MPO activity and the MDA, nitrite/nitrate, TNF-α, IL-1β, and IL-6 levels in the T/HS group were significantly increased. Acute lung injury was associated with a higher degree of pulmonary HIF-1α and iNOS expression as well as apoptosis in the lungs. Intratracheal delivery of HIF-1α inhibitor YC-1 (1 mg/kg) significantly attenuated lung injury, and reduced pulmonary HIF-1α and iNOS expression and HIF-1α transcriptional activity in the T/HS group.

Conclusion: Local inhibition of HIF-1α by YC-1 alleviates the lung injury induced by T/HS. Our results provide novel insight into the pathogenesis of T/HS-induced ALI and a potential therapeutic application.

Keywords: trauma; hemorrhagic shock; acute lung injury; hypoxia inducible factor-1α; YC-1; inflammation

Introduction

Trauma is the leading cause of death for those under 45 years of age in the United States[1]. Shock resulting from trauma and severe hemorrhage (trauma/hemorrhagic shock, T/HS) is a systemic inflammatory response that can result in multiple organ dysfunction syndrome (MODS). MODS is preceded by acute lung injury (ALI) in 83% of cases[2]. However, the cellular and molecular bases of ALI in the setting of T/HS are poorly defined. Many mechanistic studies have contributed to the development of several hypotheses, one of which is the gut hypothesis of MODS. A key element in the gut hypothesis of MODS is that a splanchnic ischemia-reperfusion (I/R) insult leading to gut inflammation and loss of barrier function is the initial triggering event that leads to MODS[3]. Currently, the majority of the molecular and cellular studies investigating T/HS-induced lung injury have focused primarily on the production of proinflammatory mediators. However, because the induction of many of these factors is secondary to or accentuated by hypoxia/ischemia, it seems likely that the molecular response triggered by the ischemic component of an I/R insult is a critical step in initiating the events that lead to the development of lung injury and MODS.

The cellular response to hypoxia or ischemia has been shown to be primarily regulated by hypoxia inducible factor (HIF)-1[4]. HIF-1 is composed of two subunits: HIF-1α and HIF-1β. HIF-1β is constitutively expressed and unaffected by hypoxia, whereas HIF-1α is continuously synthesized and
is destroyed under conditions of normoxia, as a result of its ubiquitination and subsequent degradation by the proteasomal system after hydroxylation\(^8\). HIF-1 activity is primarily regulated by the abundance of the HIF-1α subunit. Under hypoxic conditions, HIF-1α is stabilized and translocates into the nucleus where it dimerizes with HIF-1β and transactivates downstream target genes containing hypoxia-response elements (HRE) within their promoter or enhancer elements\(^6\). This master regulator of cellular and systemic oxygen homeostasis can directly regulate over 70 genes operating in all cells in response to hypoxia.

The accepted role of HIF-1α is that it acts as an adaptive and survival factor for cells exposed to hypoxia or cells undergoing stress, such as that caused by ischemic injury. However, under some circumstances, HIF-1α may be deleterious because of its ability to augment both apoptotic and inflammatory processes\(^7\). In LPS-induced ALI, HIF-1α expression exacerbates acute lung epithelial cell injury and the expression of the BCL2/adenovirus E1B 19-kDa protein-interacting protein 3 (BNIP3) gene and many proinflammatory cytokines\(^11\). Moreover, it has been shown that partial HIF-1α deficiency (HIF-1α\(^{-/-}\)) could alleviate lung injury induced by T/HS or superior mesenteric artery occlusion (SMAO) in mice\(^12\). In this study, we hypothesized that lung HIF-1α activation could be potentially maladaptive or injurious and could contribute to lung injury induced by T/HS. YC-1 [3-(5'-hydroxymethyl-2'-furyl)-1-benzyl indazole], an inhibitor of HIF-1α, has been widely used as a pharmacologic tool for investigating the physiologic and pathologic roles of HIF-1α\(^14\). Using YC-1, we tested the functional significance of HIF-1α in a T/HS-mediated lung injury model.

Materials and methods
Rats and the T/HS model
Adult male Wistar rats were purchased from the Animal Center of the Shanghai Jiao Tong University School of Medicine (Shanghai, China) and housed in air-filtered temperature-controlled units with access to food and water ad libitum. The experimental protocols were approved by the institutional animal care committee and complied with National Institutes of Health guidelines for animal experimentation. The rats were allowed to stabilize, and they were then randomized to one of four groups: (1) Sham; (2) T/HS; (3) T/HS+vehicle (dimethyl sulfoxide, DMSO); (4) T/HS+YC-1 (1 mg/kg diluted in DMSO, delivered intratracheally with a 22-gauge intubator 10 min before operation). Because DMSO has a sticky consistency, it was dissolved in phosphate-buffered saline (PBS) to prevent it from interfering with the respiration of the rats. The final concentration of DMSO was 1% for the intratracheal challenge, as described previously\(^15\). The dosage of YC-1 administered in this study was based on previous I/R studies\(^17\) and on our preliminary experiments showing that this dosage improved the outcome of T/HS in rats. For the T/HS model, the rats were anesthetized with pentobarbital (40–50 mg/kg, ip), and a 2.5-cm midline laparotomy was performed under strict asepsis. Anesthesia was maintained with pentobarbital sodium (12.5 mg·kg\(^{-1}\)·h\(^{-1}\)). Blood was withdrawn from the jugular vein until a mean arterial pressure (MAP) between 35 and 40 mmHg was obtained and maintained for 60 min. After 60 min, the rats were resuscitated with their shed blood for 3 h. Sham-shock animals underwent cannulation of the femoral artery and jugular vein followed by a laparotomy; however, no blood was withdrawn, and the MAP was kept within normal limits.

Water content determination and histologic examination
To evaluate the severity of the acute lung injury, the establishment of pulmonary edema was confirmed by the wet/dry weight ratios of the lungs. The lungs were removed, blotted dry and weighed. They were then incubated at 60°C for 72 h and reweighed. The difference between the wet weight and the dry weight was considered to be the water weight and was calculated as the percentage of the wet tissue weight. The morphologic alterations in the lungs were examined in individual rats from each of the four groups. The lungs were fixed with 4% paraformaldehyde and embedded in paraffin. Paraffin sections that were 4 μm thick were stained with hematoxylin and eosin (H&E) for examination by light microscopy. A scoring system to grade the degree of lung injury was employed\(^19\). Simultaneously, gut injury was assessed according to the grading systems for villous injury as described previously\(^12\). The grading was performed by a blinded pathologist. Injury scores were calculated by adding the individual scores for each category.

Collection of bronchoalveolar lavage fluid
After resuscitation for 3 h, the rats were sacrificed by exsanguination. The trachea was exposed and lavaged 3 times with 1 mL of PBS with a 20-gauge catheter. The lavage fluids were pooled and then centrifuged at 300×g for 5 min at 4°C. The resultant supernatants were stored at -80°C for subsequent measurements. The pellets were resuspended in PBS to determine the total and differential cell counts of the bronchoalveolar lavage fluid (BALF). The total cell count was measured with a hemocytometer. The differential cell count was determined by manually counting 200 cells per mouse that were stained with Diff-Quick (Pusheng Biological Corporation, Shanghai, China) and fixed on glass slides.

Detection of malondialdehyde (MDA) levels and myeloperoxidase (MPO) activity in lung tissue
Lipid peroxidation as a result of I/R is one of the main causes of lung injury\(^19\). The MDA levels in the tissue samples were determined as an indicator of lipid peroxidation. The absorbance of the supernatant was measured by spectrophotometry at 515–553 nm. The concentration was expressed as nanomoles per milligram of protein in the tissue homogenate. Myeloperoxidase (MPO) activity was determined as an index of neutrophil accumulation in the lungs. As described previously\(^12\), the MPO activity in the supernatants was determined by measuring the H\(_2\)O\(_2\)-mediated oxidation of o-dianisidine hydrochloride at 460 nm and normalizing relative to the num-
ber of milligrams of protein determined by bicinchoninic acid protein assay (Pierce, Rockford, IL, USA).

**Determination of pulmonary nitrite/nitrate, tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6**

At 3 h after resuscitation, the left lungs from mice in each group (n=6 for each group) were removed and snap frozen in liquid nitrogen and then stored at -80°C for subsequent analysis. To determine the nitric oxide (NO) concentrations in collected samples, we chose to measure the sum of the stable NO metabolites, the nitrite concentration and the nitrate concentration using chemiluminescence as described previously[20]. The TNF-α, IL-1β, and IL-6 concentrations were measured using a commercially available enzyme-linked immunoassay kit (ELISA) kit according to the manufacturers’ instructions (R&D systems, Minneapolis, MN, USA).

**Real-time quantitative polymerase chain reaction (PCR)**

Real-time quantitative PCR estimation of the mRNA levels was performed as previously described[21]. Briefly, total RNA was prepared from the lung tissue using RNeasy (Qiagen, Shanghai, China), and cDNA was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA) according to the manufacturer’s protocols. The cDNA was then amplified by PCR using the TaqMan gene expression Master Mix and predesigned TaqMan probes for murine HIF-1α and inducible nitric oxide synthase (iNOS) as recommended by Applied Biosystems. Within each experimental group, the mRNA expression was normalized relative to the amplification of 18S rRNA. The value determined for the sham group was set as 100%, and the levels obtained for the other groups are represented as fold over sham.

**Western blotting assay**

As described previously, total p42/p44 was used as a loading control because the expression of commonly used loading controls, such as β-actin and tubulin, has been shown to be altered in the tissues of animals subjected to I/R injury[22]. Western blotting assays were performed as we have described previously[23]. The intensity of each band was quantified using Quantity One-4.2.3 software (Bio-Rad, Hercules, CA, USA) and normalized relative to the total level of p42/p44 by density analysis.

**Electrophoretic mobility shift assay (EMSA)**

Nuclear extracts were prepared from lung tissue using a nuclear extraction kit according to the manufacturer’s instructions (Pierce, Rockford, IL USA), and aliquots were incubated with γ-32P-ATP-labeled oligonucleotides encompassing the binding site for HIF-1α (5’-TCT GTA CGT GAC CAC ACT CAC CTC-3’) (TaKaRa, Dalian, China). The EMSA was performed as previously described[23].

**Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay**

Apoptotic cells were detected and quantified by the TUNEL assay using the peroxidase (POD) in situ cell death detection kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer’s instructions.

**Statistical analysis**

The results were expressed as the mean±SEM. The differences among the groups for all variables except the pathological scores were evaluated with one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test. For the pathological scores, the differences were evaluated using the Kruskal-Wallis rank test. The results were considered statistically significant when the P value was less than 0.05.

**Results**

**Pathological changes to the lung and intestine**

As a consequence of T/HS, severe lung injury was observed, as indicated by the presence of extensive interstitial edema, infiltration of leukocytes and red blood cell congestion in the lungs of the T/HS rats (Figure 1A). These changes were significantly ameliorated in the T/HS+YC-1 rats. No evidence of lung injury was seen in the sham group. Because the gut is a major source of factors that contribute to the development of a systemic inflammatory state during acute lung injury[3], we sought to assess the intestinal injuries induced by T/HS. As shown in Figure 1C, the intestinal tissues were obviously damaged by edema, hemorrhage, and cell infiltration in the T/HS group. There was a significant difference between the T/HS group and the controls with respect to the pathological scores of the intestines and lungs pathological scores (Figures 1B and 1D). In the T/HS+YC-1 group, the pathological scores for the intestine and lung tissues were significantly lower than those of the T/HS group, suggesting that YC-1 ameliorates intestinal and lung injury induced by T/HS.

**YC-1 attenuates the infiltration of inflammatory cells**

To determine whether inhibition of HIF-1α by YC-1 affected the T/HS-induced infiltration of inflammatory cells into the airways and parenchyma, we counted the inflammatory cells in the BALF after resuscitation. As shown in Figures 2A–2D, the total numbers of inflammatory cells (including macrophages and neutrophils) and lymphocytes in the BALF were significantly higher in rats in the T/HS group compared to those in the sham group. Moreover, the increase in these cell populations in the T/HS group was significantly attenuated in the T/HS+YC-1 group.

**YC-1 attenuates pulmonary edema, MPO activity and the levels of MDA, nitrate and nitrite**

As shown in Figure 3A, rats subjected to T/HS demonstrated a significantly higher lung water content than the sham-operated animals (P<0.05). YC-1 administration significantly decreased the T/HS-related pulmonary edema (P<0.05 vs T/HS group). The MPO activity and the MDA level in the T/HS group were significantly higher than those in the sham group (P<0.05). Compared with the T/HS group, the MPO activity and the MDA level in the T/HS+YC-1 group were
markedly reduced ($P<0.05$) but were still higher than those in the sham group ($P<0.05$) (Figures 3B and 3C). As seen in Figure 3D, the mean nitrate and nitrite levels in the T/HS group were significantly higher than those in the sham group ($P<0.05$). T/HS resulted in an approximately 4-fold increase in the nitrate and nitrite levels in lung tissues. YC-1 admin-
istration significantly decreased the nitrate and nitrite levels compared with rats in the T/HS group ($P<0.05$).

**YC-1 attenuates pulmonary TNF-α, IL-1β, and IL-6 levels**

Compared with the sham group, T/HS induced a significant elevation of pulmonary TNF-α, IL-1β, and IL-6 levels. YC-1 ameliorated the accumulation of TNF-α, IL-1β, and IL-6 induced by T/HS. The values were significantly lower than those resulting from T/HS but still higher than the values in the sham group (Figures 4A–4C).

**YC-1 attenuates pulmonary HIF-1α, iNOS expression, and HIF-1α transcriptional activity induced by T/HS**

As shown in Figure 5A, real-time PCR analysis demonstrated a modest increase in HIF-1α expression in rats subjected to T/HS compared with their sham counterparts. T/HS was also capable of inducing the expression of iNOS (Figure 5B). We chose to characterize the iNOS response because iNOS has been identified as an HIF-1α target and is a key effector in the pathophysiology of acute lung injury during shocked states. In agreement with earlier studies, the baseline levels of iNOS mRNA were significantly lower in the sham group relative to those in the T/HS group. Furthermore, negligible HIF-1α and iNOS protein levels were detected in the sham group, and both HIF-1α and iNOS protein levels were increased in the lungs of rats after T/HS compared with the sham-operated rats (Figures 5C–5F). YC-1 ameliorated the induction of HIF-1α and iNOS expression by T/HS as determined by real time PCR and Western blot analysis (Figures 5A–5F). Moreover, YC-1 reduced the transcriptional activity of HIF-1α induced by T/HS as demonstrated by EMSA (Figure 6A).

**YC-1 attenuates lung epithelial cell apoptosis after T/HS**

We next investigate whether YC-1 has an effect on lung apoptosis induced by T/HS. TUNEL staining indicated that T/HS resulted in significant apoptosis of alveolar epithelial cells, while YC-1-treated rats displayed a drastic reduction of
TUNEL-positive cells (Figure 6B). Apoptotic cells were not observed in the lungs of rats in the sham groups. In agreement with the increased number of apoptotic cells, the level of the cleaved form of caspase-3, an important member of the apoptotic pathway, was decreased in YC-1-treated rats compared with T/HS rats (Figure 6C).

**Discussion**

Recent data show that the HIF-1α+/– genotype could alleviate lung injury induced by T/HS or by superior mesenteric artery occlusion (SMAO) in mice[12, 13]. However, gene deletion studies have established that HIF-1α is indispensable during fetal development, as HIF-1α–/– mice die mid-gestation owing to defects in VEGF expression and vascularization[26]. In addition, HIF-1α+/– mice manifest impaired responses to hypoxia compared with their wild-type (WT) littermates[27]. Therefore, local application of a HIF-1α inhibitor may prevent interference with the global functions of HIF-1α. In the present study, we demonstrated that multiple indicators of lung injury, including histologic changes, apoptosis in the lung, pulmonary edema, cell counts in the BALF and pulmonary neutrophil accumulation, were substantially ameliorated by YC-1 in T/HS rats. YC-1 also abrogated the induction of pulmonary inflammatory cytokines and lung apoptosis in T/HS rats. Furthermore, we demonstrated that local application of YC-1 inhibited the activation of HIF-1α in the lungs, resulting in attenuation of the activation of the iNOS-NO pathway by T/HS. Therefore, our findings suggest a critical role for HIF-1α signaling in lung inflammatory injury triggered by T/HS.

The T/HS model (laparotomy plus 60 min of hemorrhagic shock at 35–40 mmHg) and 3 h of reperfusion represents a global I/R injury, and the gut is particularly susceptible to I/R injuries[28]. The mechanisms of lung injury after T/HS are complex and have been difficult to unravel. It has been shown that damage to the intestinal mucosal barrier following T/HS causes the dislocation of bacteria, resulting in systemic inflammatory reactions[29]. I/R involves the release of a large number of inflammatory mediators, including TNF-α, IL-1β, IL-6, and NO. The neutrophils and their enzymatic products are sequestered in the lung tissues, causing increased microvascular permeability and pulmonary edema[30, 31].

HIF-1α has emerged as a critical determinant in the pathophysiological response to I/R in conditions such as...
nitrate and nitrite levels were increased significantly in T/HS-treated animals. However, YC-1 significantly inhibited the T/HS-induced increases in pulmonary nitrate and nitrite levels. The results showed that YC-1 significantly reduced the generation of NO accompanied by the down-regulation of iNOS expression. Consistent with our findings, other studies have shown that HIF-1-induced iNOS mediates neuronal cell death in astrocytes[39]. Furthermore, recent studies have demonstrated increased caspase-3 protein levels and activity are regulated via iNOS[35, 36]. Accordingly, we found that YC-1 attenuated caspase-3 accumulation and lung epithelial cell apoptosis in T/HS rats. Therefore, HIF-1-induced increases in iNOS expression are, at least, partly responsible for the injurious effect of T/HS on the lungs. It has been suggested that the superoxide ions react with NO to produce peroxynitrite, which then causes accentuated lipid peroxidation, as well as protein and DNA modifications that result in cellular damage[40]. Thus, although HIF-1α appears to be involved in the regulation of lung homeostasis, it also appears to have dichotomous roles in lung inflammatory diseases in that it can be injurious or protective depending on the exact physiological conditions studied as well as the nature and duration of the insult.

Although YC-1 has been shown to be a HIF-1α inhibitor, our knowledge of the effects of YC-1 on HIF-1α gene expression is limited and to some extent controversial. For example, in contrast to the reduction in HIF-1α mRNA expression caused by YC-1 in T/HS rats in the current study, the level of HIF-1α mRNA was nearly unchanged in the presence of different concentrations of YC-1 in hypoxic PC-3 cells[42]. One explanation for this discrepancy may be that increased transcription of the HIF-1α gene was important for HIF-1α protein induction under nonhypoxic conditions[43], as shown in our T/HS model. However, hypoxia did not induce the expression of HIF-1α mRNA in PC-3 cells, as was also the case in other cell models[44]. It could be argued that our present data have demonstrated only the preventive effects of YC-1 in ALI, and thus the therapeutic role of YC-1 in ALI remains unknown. Although we did not apply YC-1 after the development of ALI in this study, previous studies have shown that when reduced levels of HIF-1α were evident at 30 min, similar reductions were also evident at 4 and 24 h after an insult[45, 46]. Based on those studies, it would appear that the effects of YC-1 on TH/S would be expected to persist after the insult.

It should be noted that although it is well accepted that YC-1 is an effective HIF-1α inhibitor, it was originally characterized as a cGMP inducer because it stimulates soluble guanylyl cyclase activation in response to nitric oxide or carbon monoxide in some cells[47]; only low concentrations of YC-1 (1–20 µmol/L) are required for anti-HIF-1α activity, whereas cGMP elevation requires higher concentrations (>50 µmol/L)[48, 49]. It has been reported that no serious toxicity is observed in nude mice treated with YC-1 over a 2-week period[46]. Nonetheless, whether and to what extent cGMP may play a role in the beneficial effects of YC-1 in ALI induced by T/HS, particularly in relation to the inhibitory effects on HIF-1α, remains to be

Figure 6. The effect of YC-1 on HIF-1α transcriptional activity and T/HS induced lung apoptosis. (A) HIF-1α transcriptional activity in the lung of rats subjected to T/HS as evaluated by EMSA. Autoradiograph is representative of three separate experiments. (B) A representative lung section stained with TUNEL from six animals of each group is shown. (a) sham, (b) T/HS, (c) T/HS+vehicle, (d) T/HS+YC-1, original magnification×400. (C) Protein extracts of whole lung were immunoblotted for cleaved caspase-3.

Cerebral and myocardial ischemia, and its activation is an early component of the inflammatory response[32]. It has been documented that HIF-1 regulates iNOS expression[33, 34], and iNOS-derived NO is involved in the pathogenesis of lung injury[35, 36]. Recently, we found that LPS treatment induced the iNOS-NO pathway during the development of ALI[37]. In the present study, we examined the pulmonary levels of nitrate and nitrite, the oxidative metabolites of NO, to estimate NO production after T/HS injury. The pulmonary
determined.

Although our findings suggest that HIF-1α inhibition attenuates the T/HS-induced gut-and lung-derived inflammatory response and lung apoptosis, a more extensive analysis of the HIF-1-driven lung inflammatory response is needed to resolve this issue. One limitation of this study was the fact that this was purely a pharmacologic study and that only one dose of the drug was used. Consequently, genetic-based studies should be carried out to complement the pharmacologic work reported here. For example, siRNA knockdown approaches and the use of HIF-1α conditional knockout mice could be used to validate as well as extend this work. This study shows that local inhibition of the HIF-1α signaling pathway attenuates T/HS-induced lung injury and inflammation, and it provides novel insights into the biology of this clinically important disease as well as potential therapeutic insights. These results are of potential clinical importance because T/HS has been associated with the development of MODS as well as worse clinical outcomes in severely injured and intensive care unit patient populations.

In our current study, local administration of YC-1 reduced lung and intestinal injury in the T/HS model. Two possibilities exist that may explain this apparent discrepancy with the “gut hypothesis”. One possibility is that there is a feedback cycle between lung injury and intestinal injury during T/HS. Although injury to the gut is the initial triggering event in MODS, lung injury can also accelerate intestinal injury. It has been found that local acid aspiration can lead to systemic organ injury\cite{50}. Another possibility is that the “gut hypothesis” has not been conclusively shown to play a role in the expression of cytokines or tissue injury in an animal model of T/HS. Many research groups have reported an absence of endotoxemia after T/HS\cite{52,53}. As a result, our observation that intratracheal administration of YC-1 can attenuate intestinal injury induced by T/HS highlights the concept that the lungs can be a target as well as a producer of organ injury. To our knowledge, the current work shows that the local inhibition of HIF-1α can specifically limit T/HS-induced lung and intestinal injury.

In conclusion, the present study indicates that the local inhibition of HIF-1α has a protective role in lung injury induced by T/HS, and this protection may be related to HIF-1α’s regulation of the iNOS-NO pathway in lung tissue. The significant attenuation of T/HS-related lung injury when YC-1 was administered warrants further studies to improve our understanding of the specific roles of HIF-1α in T/HS-induced organ injury and to elucidate its potential therapeutic target in clinical settings.

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Author contribution

Hong JIANG and Yan HUANG performed the research; Hui XU contributed new analytical reagents and tools; Rong HU analyzed data; Qi-fang LI designed the project and wrote the manuscript.

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