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Effect of 3–3'diindolylmethane (DIM) on Acute Radiation Lung Injury in Mice Via the TGF-β1 Pathway

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Abstract: *Objective*: We studied the effect of 3–3'diindole methane (DIM) on acute radiation-induced lung injury (RILI) in mice and the possible underlying mechanism. *Methods*: A total of 45 mice were divided into five groups using the random number table method, namely, blank group, drug group alone (75 mg/kg DIM by peritoneal perfusion), simple irradiation group (one-time irradiation of 16 Gy), irradiation + drug group (one-time irradiation of 16 Gy + intraperitoneal perfusion of 75 mg/kg DIM 30 min before irradiation), and irradiation + prednisone group (one-time irradiation of 16 Gy + intraperitoneal perfusion of 5 mg/kg prednisone 30 min before irradiationas a positive control group). The whole lung was irradiated with a single dose of 16 Gy X-ray. Mice were killed by cervical dislocation at 24 h, 1 week, 2 weeks, and 4 weeks after irradiation, following which the lung tissue samples were subjected to hematoxylin and eosin (HE) staining. In addition, the expression of transforming growth factor (TGF)-β1/vascular endothelial growth factor (VEGF) pathway-related proteins was studied. *Results*: Under the same irradiation dose, the degree of lung injury in model mice after the intervention of DIM drugs was significantly lower than that in mice in the simple irradiation group. DIM significantly reduced the expression of TGF-β/VEGF-1 ($P< 0.05$). *Conclusion*: 3-3'diindolyl methane (DIM) can be downregulated by TGF-β1 signaling pathway, thereby reducing the expression of VEGF in lung tissue, inhibiting radiation-induced oxidative stress and inflammatory factor release in mouse lung tissue, and reducing the degree of RILI. These prospective experimental research results provide necessary experimental and theoretical basis for the application of 3-3'diindolemethane and its derivatives as new radiation protective drugs in clinical tumor radiotherapy.

Keywords: 3–3'diindolylmethane (DIM), TGF-β1/VEGF Pathway, Mice, Radiotherapy, Radiation-Induced Lung Injury

1. Introduction

Radiation-induced lung injury (RILI) is one of the common complications of chest radiotherapy. It is defined as an aseptic inflammatory response in normal lung tissues following radiotherapy for chest malignant tumors. Most researchers [1, 2] believe that the occurrence of RILI is intricately related to cytokine cascade, a waterfall effect of different effector cells in the lungs that release numerous cytokines and factors involved in local injury and inflammation. The most common cells are lung type II and endothelial cells that releases everal pro-inflammatory cytokines (interleukin [IL]-1a, IL-6, tumor necrosis factor [TNF]-α). Cytokines that promote tissue repair and organ fibrosis include fibrotic factors (transforming growth factor [TGF]-β and platelet-derived growth factor [PDGF]) that are released by alveolar macrophages. At present, effective intervention methods are unavailable for RILI, with only a few effective drugs reported in the literature, which are still in the preclinical stage. Therefore, it is highly crucial to identify novel protective agents for radioactive lung injury. 3–3' Diindolylmethane (DIM) is a derivative of indole-3-carbinol (I3C) and is rich in cruciferous vegetables, such as common cauliflower, cabbage, radish, and kale. It is a small molecule compound with good water solubility. Clinical trials have demonstrated that DIM can be absorbed smoothly orally and is well tolerated in humans, with no toxic side effects [3]. Studies have reported that I3C and its primary metabolite DIM can positively regulate the expression and phosphorylation of ATM and BRCA1, and closely related proteins associated with ionizing radiation-induced DNA damage and repair [4]. DIM is activated by ATM/BRCA1 phosphorylation to selectively enhance the radiation tolerance of normal tissues and prolong

the survival time of exposed animals without affecting the radiotherapy sensitivity of tumor tissues, suggesting that DIM could serve as a potential radio protective candidate with strong selectivity and good efficacy [5]. Therefore, we constructed a mouse RILI model by single whole chest irradiation of 16Gy to investigate whether DIM affected mouse RILI by regulating the TGF-β1/VEGF signaling pathway and provide a new basis and direction for the therapeutic application of DIM in RILI.

2. Materials and Methods

2.1. Materials

2.1.1. Experimental Drugs

3–3'Diindolylmethane (DIM, 3,3'-diindolylmethane) was purchased from Jiangsu Pulusi Biotechnology Co., Ltd., molecular formula C17H14N2 (Figure 1), molecular weight 246.3065, purity >99%.

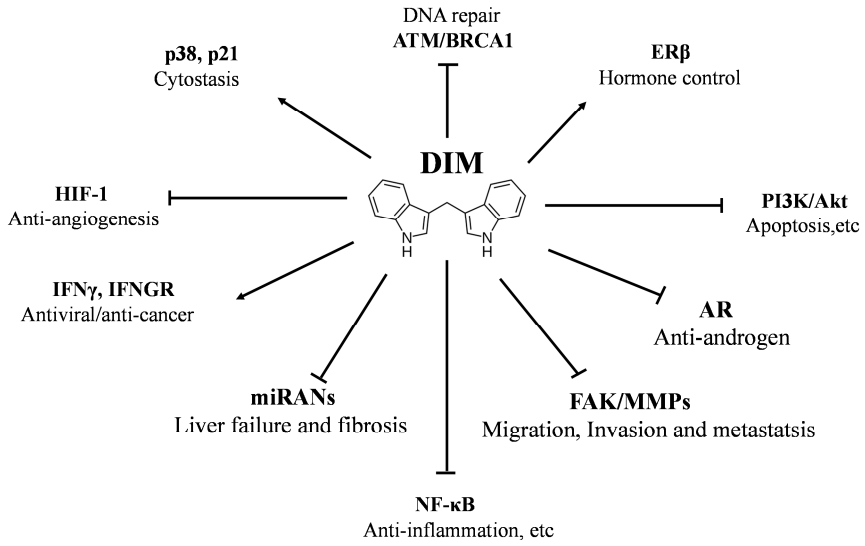


Figure 1. *Chemical structure of 3,3'-diindolylmethane.*

2.1.2. Laboratory Animals

Forty-five clean-grade C57BL/6 mice weighing 20 to 22 g were purchased from the Wuxi Jiangnan Laboratory Animal Center. The animals were kept at room temperature ranging from 23 to 25°C, standard humidity of 55% to 60%, and 12 h light. They were fed food and water *ad libitum* threedays later for experiments.

2.1.3. Laboratory Equipment and Reagents

(i). Laboratory Equipment (Table 1)

Table 1. *Laboratory Equipment.*

Name	Manufacturer	Model
Dehydrator	Wuhan Junjie Electronics Co., Ltd	JJ-12J
Embedding machine	Wuhan Junjie Electronics Co., Ltd	JB-P5
Pathological slicer	Shanghai Leica Instrument Co., Ltd	RM2016
Frozen platform	Wuhan Junjie Electronics Co., Ltd	JB-L5
Organize the spreader	Zhejiang Jinhua Kedi Instrument Equipment Co., Ltd	KD-P
Roaster	Tianjin Leborui Instrument Equipment Co., Ltd	GFL-230
Slide	BioBMW	
Upright light microscope	Nikon, Japan	Nikon Eclipse E100
Imaging system	Nikon, Japan	Nikon DS-U3

(ii). Experimental Reagents (Table 2)

Table 2. *Experimental Reagents.*

Reagent name	Manufacturer	Article No.
Anhydrous ethanol	Sinopharm Chemical Reagent Co., Ltd.	100092683
Xylene	Sinopharm Chemical Reagent Co., Ltd.	10023418
HEdye liquor	BioBMW	G1005
Differentiation fluid	BioBMW	G1005–3
Blue-return liquid	BioBMW	G1005–4
Neutral balsam	Sinopharm Chemical Reagent Co., Ltd.	10004160

2.2. Methods

2.2.1. Animal Grouping

Forty-five C57BL/6 mice were randomly divided into a blank group (NC group), drug-only group (DIM group, DIM group, DIM by intraperitoneal perfusion, 75 mg/kg), irradiation group alone (RT group, one-time irradiation of 16Gy), irradiation + drug group (RT + DIM group, one-time irradiation of 16Gy + peritoneal perfusion DIM 30 min before irradiation, 75 mg/kg), irradiation + prednisone group (RT+PDN group, one-time irradiation of 16 Gy + 30 min before irradiation by intraperitoneal perfusion of 5 mg/kg prednisone as a positive control group).

2.2.2. Model Making

Mice were anesthetized intraperitoneally using 0.2 mL of 0.5% pentobarbital. Mice in the simple irradiation group, irradiation + drug group, and irradiation + prednisone group were single-irradiated with accelerator 6MV-X-rays at a dose of 16Gy; 75 mg/kg DIM was administered via intraperitoneal perfusion. Mice in the irradiation + drug group were perfused peritoneally with 75 mg/kg DIM 30 min before irradiation. Mice in the irradiation + prednisone group were perfused peritoneally30 min before irradiation with 5 mg/kg prednisone. Those in theblank group were administered the same dose of normal saline gavage. After anesthesia, the limbs of the mice were fixed with clips, they were placed on a medical linear accelerator treatment bed, and the laser was positioned on the whole chest. A1 × 2 cm irradiation field was set with a source skin distance of 98 cm. After irradiation, mice were returned to the original place for rearing, and their general condition was recorded after irradiation. Mice were sacrificed at four time points, namely 24 h, 1 week, 2 weeks, and 4 weeks after irradiation. One was executed in the blank group and six in the remaining groups. An EP tube was coated with heparin anticoagulant, and blood was collected from the eyeball, following which 100 μL of it was placed in the EP tube, shaken, and centrifuged at 5000 rpm for 2 min. Afterward, the serum was aspirated for backup. Mice were sacrificed by cervical dislocation, lung tissue was taken, fixed in 10% formalin solution, dehydrated using gradient ethanol, paraffin-embedded, and 5 μm sections were obtained. The sections were hematoxylin and eosin (HE)-stained and observed under a microscope. A small amount of tissue was placed in a cryopreservation tube and stored in liquid nitrogen. The total protein was extracted after lysis by ultrasonography, and the expression of related signaling pathway proteins was detected by western blotting. Wax blocks and pathological sections were observed to study the changes in lung tissue structure and infiltration of inflammatory cells after radiation in different groups under light microscopy.

2.3. Statistical Analysis

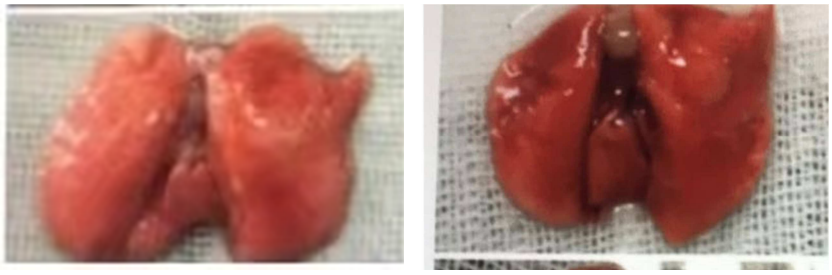
SPSS 19.0 statistical and GraphPad Prism 5.0 were used for data analysis. $P<0.05$ was considered significant.

3. Results

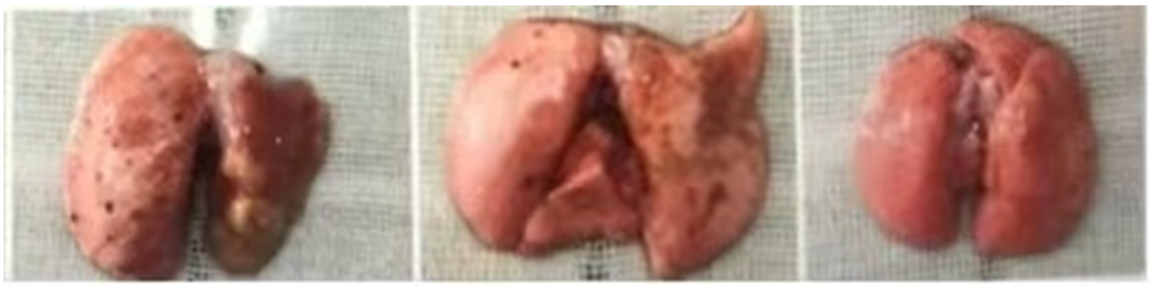
3.1. Lung Histopathology in Each Group

The results of lung morphology showed that the lung tissue of mice in the blank control group was composed of alveoli, bronchial branches, blood vessels, and interstitium, with a clear structure. However, most mice in the drug group displayed mild to severe hyperemia of alveolar walls, and some were accompanied by mild or moderate inflammatory cell infiltration. The inflammatory cell types were largely mononuclear cells, with several lymphocytes observed in the alveolar wall or around the bronchi and blood vessels. In the simple irradiation group, the alveolar wall had mild to severe hyperemia, some of which were accompanied by mild or moderate infiltration of inflammatory cells. The type and distribution of inflammatory cells were the same as before. Mild emphysema was rarely present. Morphological manifestations included large alveolar cavities, which could have been caused by rupture and fusion of the alveolar wall. The lung tissue structure of mice in the irradiation + drug group was the same as those in the blank control group, and the degree of severity of the lesion was reduced compared with that in the simple irradiation group. The lung tissue structure was the same as that of the blank group, with a reduced number of lesions compared with the model group (Figure 2).

The results of HE staining revealed a complete alveolar structure, a thin alveolar wall, no septal edema, and inflammatory cell infiltration in the control group. In this experiment, the chest irradiation of BALB/c female mice caused radiation-induced lung damage, primarily manifested as alveolar wall hyperemia and inflammatory cell infiltration, with the inflammatory cell type being largely mononuclear cells. Lymphocytes were observed. Compared with the degree of severity of lesions in each group, those in the simple irradiation group were the most evident, followed by the irradiation + strong pine group, whereas the irradiation + DIM group displayed the mildest degree of lesions (Figure 3). A time-point comparison revealed that the response to RILI was evident 2 weeks after irradiation. The results were significant ($P<0.05$, Table 3).

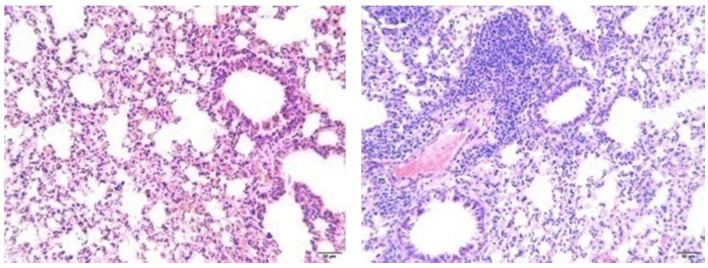


Blank control group (NC group) Drug-only group (DIM group)

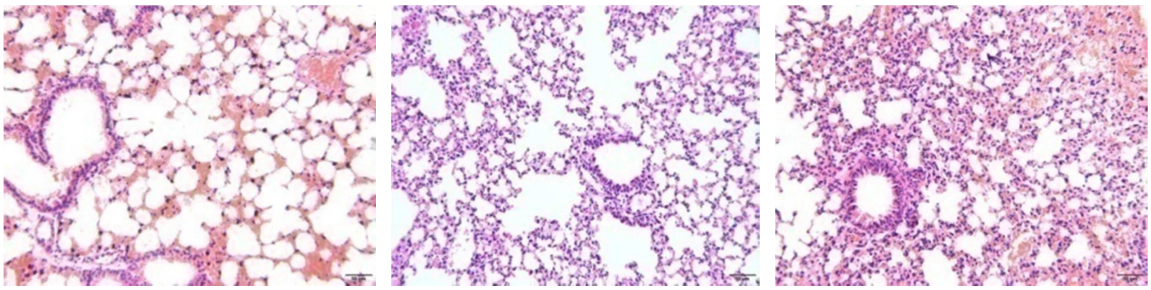


Irradiation alone group (RT group) Irradiation + prednisone group (RT+PDN group) Irradiation + DIM group (RT+DIM group)

Figure 2. *Lung morphology.*



Blank control group (NC group) Drug-only group (DIM group)



Irradiation alone group (RT group) Irradiation + prednisone group (RT+PDN group) Irradiation + DIM group (RT+DIM group)

Figure 3. *Pathological observations of lung tissue of mice in each group (HE staining, ×200) (2 weeks after irradiation).*

Table 3. *Effects of 3–3' diindolylmethane (DIM, 3,3'-diindolylmethane) on lung tissue damaged by radiation ($\bar{x}$±SD, n = 6).*

Group	24 h	1week	2weeks	4weeks
NC group	3.0 ±0.9	3.2 ±0.2	3.5 ±0.3	3.6 ±0.1
DIM group	4.3 ±0.2	4.4 ±0.5	4.2 ±0.6	4.3 ±0.3
RT group	5.2 ±1.1	5.5 ±0.4	5.9 ±0.2	5.6 ±0.5
RT+PDN group	5.0 ±0.2	5.2 ±0.5	5.4 ±0.2	5.4 ±0.1
RT+DIM group	4.7 ±0.4	4.9 ±0.4	5.1 ±0.4	4.8 ±0.4
p-Value	＜0.01	＜0.001	＜0.001	＜0.001

3.2. Comparison of Protein Expression of TGF-β1, VEGF, and GAPDH in Lung Tissues of Mice in Various Groups

Western blotting was used to detect protein expression (Figure 4). Compared with the control group, the expression of proteinsTGF-β1 and VEGF in the simple irradiation group and the irradiation + prednisone group was significantly increased (all P<0.05). Compared with the simple irradiation group, the expression of TGF-β1 and VEGF proteins in the DIM+ irradiation group was significantly reduced (both P<0.05).

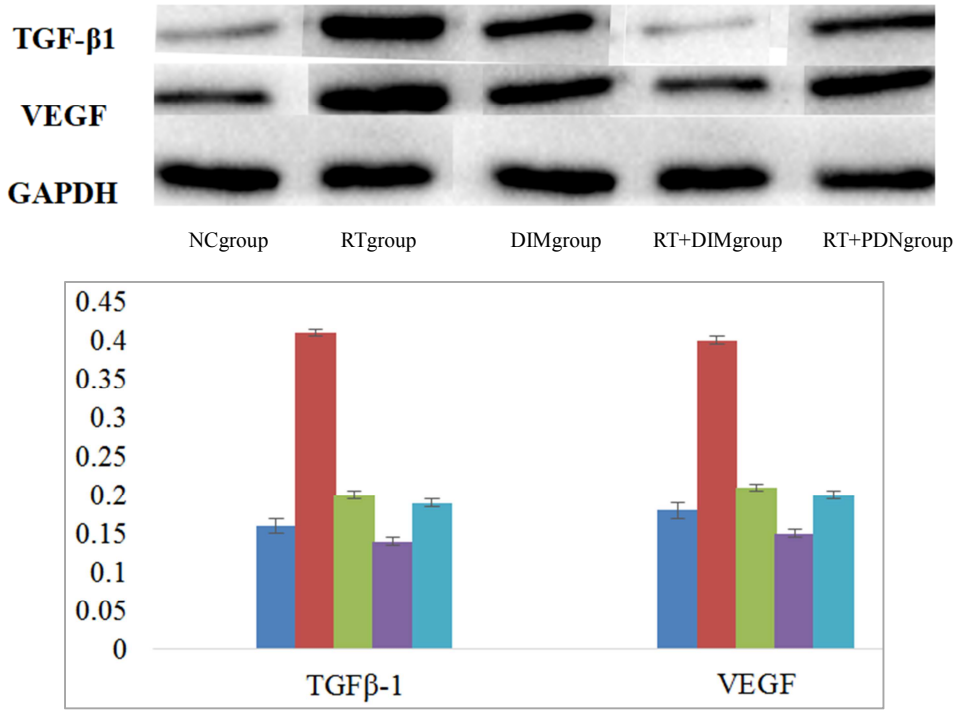


Figure 4. *Comparison of protein expression of TGF-β1, VEGF, and GAPDH in lung tissue of mice in each group.*

4. Discussion

Radiotherapy-induced lung injury includes radiation pneumonia and radiation fibrosis. These injuries are not characterized by any time boundary and comprise ionizing radiation-induced free radical generation and DNA damage, enhanced oxidative stress, vascular damage, and persistent inflammatory response that destroys alveolar type II and vascular endothelial cells. Prolonged alveolar and vascular damage leads to EMT and/or endothelial cell–mesenchymal transformation (EndoMT), ultimately causing pulmonary fibrosis changes [6]. Therefore, it is important to reduce the occurrence of RILI by pharmacological intervention to inhibit the formation of oxidative stress and accumulation of inflammatory factors in the lungs after radiotherapy.

Numerous studies have confirmed that DIM can exert anti-tumor effects through different mechanisms, such as regulating apoptosis via the phosphoinositide 3-kinase (PI3K)/Akt pathway and hypoxia-inducible factor (HIF) 1-mediated inhibition of angiogenesis [7, 8]. Studies have reported that DIM can significantly increase the sensitivity of tumor cells to radiation through BRCA1 and nuclear factor (NF)-κB signaling pathways. It is rare that DIM does not increase the side effects in normal tissues but exerts a certain protective effect, providing a basis for the application of DIM as a radiosensitizer. The results of this study were published in the prestigious journal *Proc Natl Acad Sci USA* [6]. The radiosensitizing effect of DIM was confirmed by subsequent studies [9]. Recent studies have demonstrated that orally administered DIM exerts a certain protective effect on fatal bone marrow injury induced by total body irradiation. The major underlying mechanisms include reducing the level of oxidative stress and enhancing DNA repair function [10, 11]. These studies suggest the radioprotective effect of DIM on RILI, providing a sufficient theoretical basis and preliminary basis for our experiment.

Radiation pneumonitis is a class intricately related to mediator-mediated fibrosis. Therefore, suppression of inflammation and fibrosis is believed to reduce radiation-induced lung damage. Recent literature has demonstrated that DIM exerts anti-inflammatory effects by regulating T cells [12] and alleviates the progression of liver fibrosis through miR-21 in liver injury [13, 14]. Therefore, we speculate that DIM protects against radiation pneumonia by inhibiting inflammatory response, and the process involves cycloxygenase (COX)-2 and NF-κB-related signaling pathways. As a promising γ radiation sensitizer [15], DIM can reduce hematopoietic injury caused by total body irradiation in mice [16]; however, there exists a lack of direct evidence on the preventive role of DIM to mitigate RILI, with no information on the underlying molecular mechanism. Thus, further research is warranted. In this experiment, no mice died after a single 16Gy X-ray full chest irradiation [2, 17]; compared with the normal group of mice, the major manifestations were alveolar wall hyperemia and inflammatory cell infiltration of mostly mononuclear cells. Lymphocytes were visible. These findings are consistent with those reported for the mouse radiation-induced lung injury model. Thus, the established mouse model of RILI was successful. Compared with the degree of severity of lesions in each group, those in the simple irradiation group were severe, followed by the irradiation + strong pine group, where as the irradiation + DIM group had the mildest degree of lesions. These results suggest that DIM and prednisone exert protective effects against radiation-induced lung damage.

TGF-β1 has been widely recognized as a predictor of RILI. It is primarily synthesized and secreted by epithelial cells, inflammatory cells, and mesenchymal cells [18, 19]. Its over expression has been reported to promote inflammatory response and induce oxidative stress by triggering the release of reactive oxygen species, which contribute to the occurrence and development of RILI. Inhibition of the expression of TGF-β1 is known to delay the process of pulmonary fibrosis [20, 21]. Studies have demonstrated that radiotherapy-induced pulmonary fibrosis is accompanied by vascular neogenesis, VEGF is the primary inducer of angiogenesis, and TGF-β1 can induce the production of reactive oxygen species by regulating its expression, thereby regulating the oxidative response of lung tissues [22, 23]. TGF- β 1 plays a crucial role in the process of cell apoptosis, and type II alveolar epithelial cells release TGF- β 1 leads to the occurrence of pulmonary fibrosis, while TGF- β Inducing the expression of vascular endothelial growth factor (VEGF), thereby accelerating lung fibers. The process of transformation. Research has confirmed that DIM can function through multiple mechanisms. Antitumor effects, such as regulating the PI3K/Akt pathway to increase tumor cell apoptosis Death, inhibiting angiogenesis by reducing HIF-1. The results showed that pre irradiation DIM intervention can significantly reduce lung tissue damage and significantly reduce radiation induced TGF-β1 and VEGF expression in mouse lung tissue, suggests that DIM has a radiation protective effect, which may be related to the inhibition of TGF-β1/VEGF signaling pathway is involved. DIM is expected to become a potential drug for protecting against radiation induced lung injury, but its toxicity, dosage, and mechanism of action still need further research.

Ethical Approval and Consent to Participate

Not applicable.

Availability of Data and Materials

N/A.

Publish Consent

Not applicable.

Conflicts of Interest

The author reports no conflicts of interest in this work.

Provide Funding

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Author Contribution

Qin ge contributed all to this work.

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