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Original Research Article

Guizhi-jia-houpu-xingzi decoction attenuates ovalbumin-induced allergic asthma via regulation of Toll-like receptor signal pathway

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Abstract

Purpose: *To study the effect of Guizhi-jia-houpu-xingzi (GHX) on ovalbumin-induced allergic asthma in rats.*
Methods: *An animal model of allergic asthma (AA) in rats was established by intraperitoneal injection (ip) of ovalbumin (OVA). Thereafter, GHX (375 mg/kg) was administered orally for 7 days. Pulmonary function, inflammatory cells, immunoglobulin E (Ig) E, interleukin-4 (IL)-4 and interferon-γ (IFN)-γ in serum and bronchoalveolar lavage fluids (BALF) were determined. Furthermore, mRNA expressions of Toll-like receptors (TLRs) signal pathway was determined using real time polymerase chain reaction PCR (q-RT-PCR).*
Results: *GHX (375 mg/kg) significantly decreased respiratory rate ($p < 0.01$) and Penh value ($p < 0.05$) when compared with AA rats. The inflammatory cells ($p < 0.01$) and levels of IL-4 ($p < 0.01$) and IgE ($p < 0.01$) were significantly decreased by GHX treatment when compared with AA rats; whereas IFN-γ ($p < 0.05$) was significantly increased. Furthermore, GHX significantly decreased the mRNA expressions of GATA binding protein (GATA)-3 ($p < 0.01$), TRL-2 ($p < 0.01$), TRL-4 ($p < 0.01$), myeloid differentiation factor 88 (MyD88) ($p < 0.01$), TNF receptor associated factor 6 (TRAF6) ($p < 0.01$) and β-arrestin ($p < 0.01$) in lung tissues, relative to AA rats. However, GHX treatment led to significant up-regulation of mRNA expression of T-bet ($p < 0.01$).*
Conclusion: *These results demonstrate that GHX possesses a potential for treating allergic asthma via regulation of Toll-like receptor (TLR) signal pathway. They also provide a scientific basis for the probable use of GHX in clinical treatment of allergic diseases in future.*

Keywords: *Guizhi-jia-houpu-xingzi decoction, Ovalbumin, Allergic asthma, Toll-like receptor*

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INTRODUCTION

Current epidemiologic studies have revealed that the prevalence and incidence of allergic diseases (especially allergic asthma) have increased sharply world-wide [1,2]. Allergic asthma is a complex chronic airway inflammatory reaction mediated by mastocytes, eosinophils and T-lymphocyts [3,4]. Recent reports showed that morbidity from allergic asthma is approximately 18 % in Western counties, and that there are about 300 million allergic asthma patients in the world, while over 180 thousand patients die yearly from the disease [5,6]. Therefore, allergic asthma is a serious threat to public health.

Currently available drugs for treating allergic asthma are mainly bronchodilators, anti-inflammatory drugs, leukotriene receptor antagonists and immune-modulators, such as

theophylines, immunoglobulin (Ig) E and glucocorticoids [7,8]. However, these drugs just alleviate only the symptoms of allergic asthma without providing radical cure [9]. In addition, longtime use of these drugs might result in some serious side-effects, such as gastrointestinal discomfort, drug dependency, and blood capillary injury [8,10]. Thus, it has become necessary to find some new sources of reliable drugs of low toxicity for curing allergic asthma.

Guizhi-jia-houpu-xingzi decoction (GHX) is a popular traditional Chinese formula used which produces good therapeutic effects on allergic asthma in clinics [11]. It has been reported that GHX down-regulated TNF-α in bronchoalveolar lavage fluids (BALF) of experimental asthma guinea pig, indicating that GHX may have potential benefits for allergic asthma patients [12]. However, so far, there are no systemic animal studies and its mechanism of action extensively limits the clinical application of GHX in the treatment of allergic asthma or other allergic diseases. In the present study, an experimental allergic asthma rat animal model was established induced by induction with ovalbumin (OVA). Based on the animal model, the therapeutic effects of GHX were evaluated and the possible pharmacological mechanisms were explored.

EXPERIMENTAL

Chemicals and reagents.

Ovalbumin (OVA) was purchased from Sigma-Aldrich Co. (Shanghai, China). Aminophylline (ANP) was purchased from Southwest Pharm. Ltd. (Chongqing, China). Trizol reagents were products of Invitrogen Co. (Carlsbad, CA, USA). Rat Elisa kits for secretory immunoglobulin A (sIg A), immunoglobulin E (IgE), interleukin-4 (IL-4) and interferon- γ (IFN-γ) were products of Neobioscience Co. (Shenzhen, China). HiScript 1st Strand cDNA Synthesis kits and SYBR Green Master Mix were products of Vazyme Biotech. (Nanjing, China). Hematoxylin and eosin (H & E) was purchased from the Baso Biotech. (Zhuhai, China). All primers used in the study were designed by Primer-Express v3.0 and synthesized by Sangon Biotech. (Shanghai, China, Table 1).

Animals

Male Sprague-Dawley (SD) rats (3 - 4 weeks old, 200 ± 20 g) were purchased from the Dashuo Laboratory Animal Co. Ltd (Chengdu, China) and were kept in a temperature (22 ± 2 °C) and humidity (40 – 60 %) controlled room with food and water provided *ad libitum*. All the animal experimental protocols were according to the International Principles of Laboratory Animal Care [13] and approved by the Ethics Committee for Laboratory Animal Experimentation of Chengdu University of Traditional Chinese Medicine (approval no. 2014KL-061).

Preparation of GHX extracts

All the crude TCMs in GHX were decocted with pure water. The TCMs were *Cinnamomi ramulus* (9 g), *Magnoliae officinalis cortex* (6 g), *Armeniacae semen amarum* (6 g), *Paeoniae radix alba* (9 g), *Glycyrrhizae radix rhizoma* (6 g), *Zingiberis rhizoma recens* (9 g) and *Jujubae fructus* (15g).

Table 1: Primers used

Gene		Sequence
GATA-3	Forward	5’-CTGGAGGAGGAACGCTAA-3’
	Reverse	5’-GTCTGTTAATATTGTGGAGTTTGT-3’
T-bet	Forward	5’-CCATTCCTGTCCTTCACT-3’
	Reverse	5’-CCACCAAGACTACATCCA-3’
TLR-2	Forward	5’-GTTGCGTTACATCTTGGA-3’
	Reverse	5’-GGAATACACAGTGCTCAG-3’
TLR-4	Forward	5’-CAGCTCGTTTCTCACCCAGT-3’
	Reverse	5’-TGTATCGGTGGTCAGTGTGC-3’
MyD88	Forward	5’-CGACGCCTTCATCTGCTA-3’
	Reverse	5’-GCCGATAGTCTGTCTGTTCT-3’
TRAF6	Forward	5’-CAGTCCCCTGCACATTCAGT-3’
	Reverse	5’-CTGGGCCAACAGTCTCATGT-3’
β-Arrestin	Forward	5’-GGGCATTTGTACTGAGCTGT-3’
	Reverse	5’-TGCACCTTGAGGCATCTCTG-3’
β-Actin	Forward	5’-AGGGAAATCGTGCGTGACAT-3’
	Reverse	5’-GAACCGCTCATTGCCGATAG-3’

The TCMs were purchased from Beijing *Tongrentang* TCM Chain Drug Store (Chengdu, China). They were authenticated by Professor Min Li (Department of Pharmacognosy, College of Pharmacy, Chengdu University of TCM, Chengdu, China). After decocting 3 times (each for 1 h), the total extracts were filtered and subsequently dried in vacuum at 55 °C to obtain the GHX extract (yield was approximately 10 %). The dose of GHX used (375 mg/kg) in this research was estimated from its clinical dose.

Experimental protocols and animal model preparation

A total of 40 rats were randomly divided into 4 groups (n =10): **1**) normal group, **2**) model group (control group), **3**) positive treatment group [treated with aminophylline (ANP)], **4**) GHX treatment group (375 mg/kg). Except for the normal rats, all animals in control and positive groups were treated with OVA to induce allergic asthma (AA) rats according to previously described methods with minor modifications [4]. Rats were immunized via intraperitoneal injection (*ip*) of 1 mL OVA- aluminum hydroxide mixture (1 mg OVA and 200 mg aluminum hydroxide were dissolved in 1 mL saline) on days 0 and 7. Thereafter, the rats were activated with 1 % OVA-saline solution by aerosol inhalation in a glass box (10 cm × 10 cm × 20 cm) from 14 to 21 days (30 min/day). Rats in the normal group were subjected to the same procedure, but with saline in place of OVA and aluminum hydroxide mixture. In the positive and GHX treatment rats, ANP (300 mg/kg) and GHX (375 mg/kg) were administered orally from 14th to 21st day. On day 21, pulmonary functions were determined, and after that, the rats were sacrificed under anesthesia using pentobarbital sodium (45 mg/kg, *ip*) after collection of blood samples and bronchoalveolar lavage fluids (BALF). The lung tissues were excised for the following biochemical assays [14,15].

Determination of pulmonary functions

Pulmonary functions (respiratory rate and airway hyperreactivit) were determined using a Buxco Animal Pulmonary Function analysis system (FinePointe™ NAM, Data Sciences International Inc, St. Paul, MN, USA).

Blood cell count

Blood smears were prepared and fixed with formalin. Wright-Giemsa staining was performed, and subsequently the cell counts was carried out under an optical microscope (Olympus 2H12003, Tokyo, Japan).

Assay of sIgA, IgE, IL-4 and IFN-γ by ELISA

The levels of sIgA and IgE in BALF and levels of IL-4 and IFN-γ in serum were determined by commercial ELISA kits according to the manufactures' instructions. Absorbance was read at 450 nm in a micro-plate reader (Thermo Mulliskan Ascant 413MBY042078, Waltham, MA, USA).

Real-time PCR (qRT-PCR) assay

Lung tissues obtained from each rat were separately homogenized, and total RNA extracted with Trizol reagent. Subsequently, the total RNA was used for synthesis of cDNA for GATA-3, T-bet, TRL-2, TRL-4, MyD88, β-arrestin and β-actin with reverse transcription by using qRT-PCR (CFX96TM Real-Time System, Bio-Rad, Hercules, CA, USA). The mRNA primers used in the real-time PCR experiments are shown in Table 1. Reverse transcription was performed according to the manufacturer's instructions on the commercial kits for the quantitative real-time RT-PCR reaction.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Statistically significant differences were analyzed using two-tailed Student's *t*-test using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Pulmonary function

Compared with normal rats, the respiratory rate ($p < 0.01$) and penh value ($p < 0.01$) of the allergic asthma model rats were significantly increased. However, similar to positive drug treatment, GHX (375 mg/kg) significantly reversed the increases in respiratory rate ($p < 0.01$) and Penh value ($p < 0.05$) when compared with the control rats (Figure 1).

Blood cell count

Eosinophils, neutrophils, lymphocytes and monocytes are significantly increased in the allergic asthma control rats when compared to normal rats ($p < 0.01$, Table 1). In contrast, the positive treatment reversed the increases in these inflammatory cells ($p < 0.01$, $p < 0.01$, $p < 0.05$ and $p < 0.01$, respectively). Similar to the positive drug, GHX (375 mg/kg) treatment also resulted in significant decreases in all the four

Table 2: Blood cell count (×10^6/L)

Group	Eosinophils	Neutrophils	Lymphocytes	Monocytes
Normal	2.66 ± 1.20	22.50 ± 5.50	37.00 ± 5.00	2.00 ± 0.36
Control	27.75 ± 2.19##	41.85 ± 1.92##	68.50 ± 3.52##	5.33 ± 0.88##
Positive	3.28 ± 0.47**	29.00 ± 1.15**	55.00 ± 2.30*	2.88 ± 0.26**
375 mg/kg	2.37 ± 0.37**	23.00 ± 3.60**	30.33 ± 3.77**	2.66 ± 0.88**

*Aminophylline (300 mg/kg) was used as positive drug. Data presented as mean ± SD (n = 10); *p < 0.05, **p < 0.01, vs. control rats; ##p < 0.01, vs. normal rats*

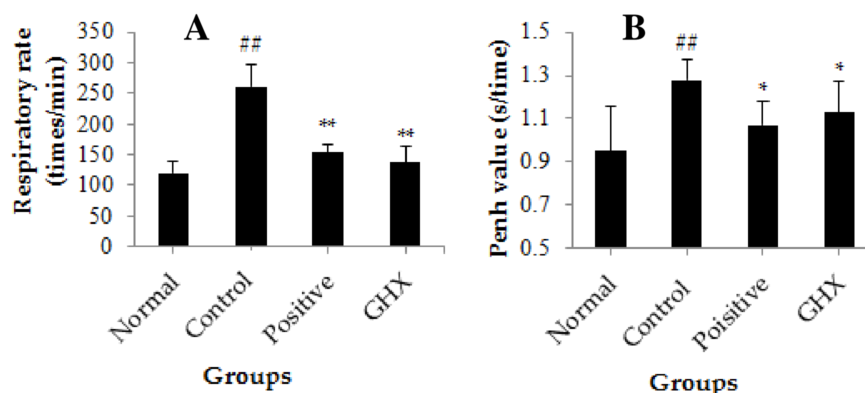

Figure 1: Respiratory rate (**A**) and Penh value (**B**). Aminophylline (300 mg/kg) was used as positive drug. Data are presented as mean ± SD (n = 10); *$p < 0.05$, **$p < 0.01$, vs. control rats; ##$p < 0.01$, vs. normal rats

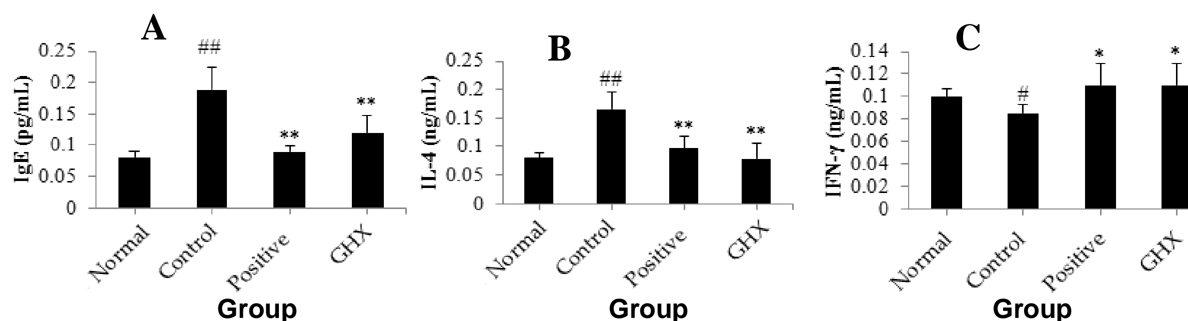

Figure 2: ELISA assays for IgE in serum (**A**), and IL-4 (**B**) and IFN-γ (**C**) in BALF. Aminophylline (300 mg/kg) was used as the positive drug. Data are presented as mean ± SD (n = 10); *$p < 0.05$, **$p < 0.01$, vs. control rats; ##$p < 0.01$, vs. normal rats

blood cell counts relative to corresponding values for control rats ($p < 0.01$).

Levels of IgE in serum and IL-4 and IFN-γ in BALF

As shown in Figure 2A, serum IgE was sharply increased ($p < 0.01$) when compared to normal rats. After treatment with positive drugs ($p < 0.01$) and GHX (375 mg/kg), level of IgE was significantly decreased ($p < 0.01$) when compared to that of control rats. After induction with OVA, the IL-4 levels (p < 0.01, Figure 2B) of control rats were significantly increased whereas IFN-γ ($p < 0.01$, Figure 2C) was significantly decreased. For the positive treated rats, the IL-4 ($p < 0.01$, Figure 2B) was significantly decreased and the IFN-γ ($p < 0.01$, Figure 2C) was significantly increased compared with the control rats. Importantly, GHX treatment showed similar results to the positive drugs, and IL-4 levels in BALF were significantly decreased by treatment with GHX (375 mg/kg) ($p < 0.01$, Figure 2B) whereas the IFN-γ levels were significantly increased by GHX at the dose of 375 mg/kg ($p < 0.05$, Figure 2C).

mRNA expressions of GATA-3, TRL-2, TRL-4, MyD88, TRAF6 and β-arrestin

As shown in Figure 3, the mRNA expressions of GATA-3 ($p < 0.01$), TRL-2 ($p < 0.01$), TRL-4 ($p < 0.01$), MyD88 ($p < 0.01$), TRAF6 ($p < 0.01$)and β-arrestin ($p < 0.01$) were significantly up-regulated in the lung tissues whereas that of T-bet ($p < 0.01$)was significantly down-regulated when compared with normal rats. Interestingly, the positive treatment and GHX (375 mg/kg) brought about significant decreases in the mRNA expressions of GATA-3 ($p< 0.01$), TRL-2 ($p < 0.01$), TRL-4 ($p < 0.01$), MyD88 ($p < 0.01$), TRAF6 ($p < 0.01$) and β-arrestin ($p < 0.01$) in

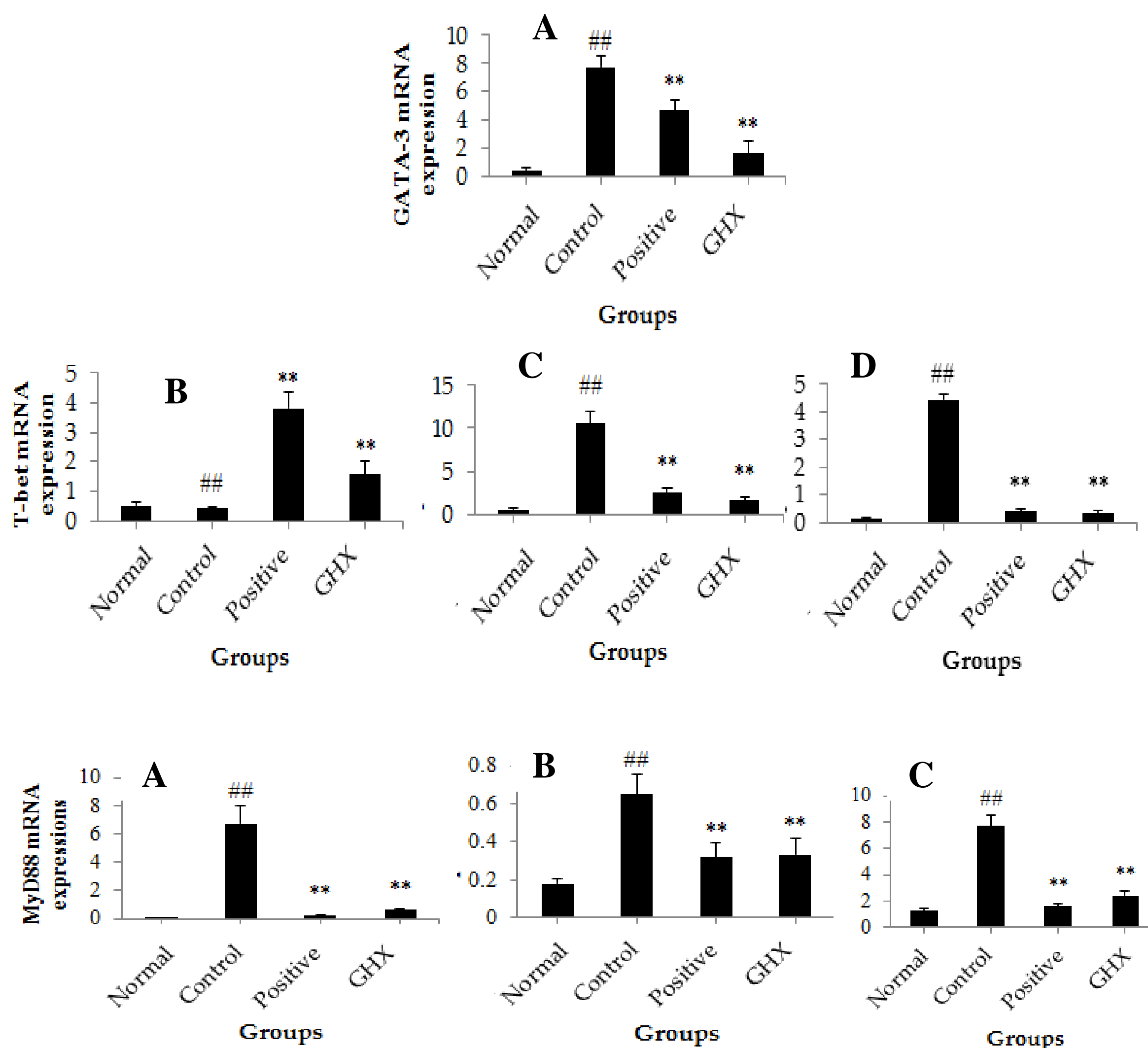

Figure 3: mRNA expressions of **(a)** GATA-3, T-bet, TLR-2 and TLR-4 and **(b)** MyD88, TRAF6 and β-arrestin. Aminophylline (300 mg/kg) was used as the positive drugs. Data presented as Mean ± SD (n = 10); ***p* < 0.01, vs. control rats; ##*p* < 0.01, vs. normal rats

lung tissues, when compared with control rats. However, mRNA expression of T-bet was up-regulated by treatment with GHX at the dose of 375 mg/kg (*p* < 0.01) relative to control rats.

DISCUSSION

The establishment of a suitable animal model is the first and most crucial step in investigating the therapeutic effects of candidate drugs [16]. In the present investigation, an allergic asthma rat animal model was successfully prepared by induction with OVA. The results showed that the model rats manifested obvious features of allergic asthma, which were reflected in the results of pulmonary functions and inflammatory blood cell counts. The results also demonstrated that GHX has potential therapeutic effect against OVA-induced allergic asthma based on the rat model.

IgE is usually expressed in allergic asthma patients, due probably to their high mucosal immune responses [17,18]. In the present research, GHX significantly decreased the serum levels of IgE in the asthmatic rats. In allergic asthma patients, infiltrated inflammatory cells over-release IL-4, which aggravates airway inflammatory reactions. In addition, IFN-γ/IL-4 ratio is a reflection of the proportion of Th1 and Th2. Increases in IFN-γ are beneficial to asthma patients. The present results show that GHX decreased the level of IL-4 and increased the level of IFN-γ in BALF [19-21]. Just like IFN-γ/IL-4 ratio, T-bet/GATA-3 ratio can also be considered a measure of Th1/Th2 cytokine profiles. Studies have shown that allergic asthma results from lymphocyte proliferation of allergen-specific type 2 T-helper (Th2) with over-release of Th2 cytokines such as IL-4, IL-5, IL-13 and IL-25 [19,22]. Thus, increases in T-bet/GATA-3 ratio would be beneficial for treating allergic asthma. Toll-like receptors (TLRs) are membrane-located

pattern recognition receptors closely related to immunocompetence [23,24]. TLRs can recognize specific conserved molecular components of microorganisms and then transfer the signals into the cell, leading to the activation of NF-κB [25,26].

The typical pathological changes in asthma are airway inflammatory reactions and airway remodeling. TLRs play important roles in the development of airway inflammatory reactions, and activation of the MyD88- IRAK-TRAF6-IKK-NF-κB signal pathway [27]. In addition, TLRs promote not only the maturity and differentiation of immune cells but also the conversion of CD^{4+} T cells into T-regulatory cells (Tr cells). Thus, TLRs-NF-κB signal pathway also regulatesTh1/Th2 ratio via Tr cells [28]. It has been reported that TLR-2 and TLR-4 are closely related to the re-organizations of peptidoglycan and lipopolysaccharides (LPS), respectively.

The β-arrestin influences the functions of T cells in several ways, and is up-regulated in allergic asthma patients [29]. The present research shows that the expressions of TRL-2, TRL-4, MyD88, TRAF6 and β-arrestin mRNAs were significantly up-regulated in lung tissues of the model rats. Interestingly, the GHX treatment significantly decreased the expressions of mRNAs of these genes in lung tissues.

CONCLUSION

The findings of this study demonstrate that GHX possesses potentials for application in the treatment of allergic asthma, most likely v regulation of TLRs signal pathway in rats. However, additional investigations are required to determine the suitability of GHX in the clinical treatment of allergic diseases.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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