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Original Research Article

Circ_0001953 contribute to retinal vascular endothelial cell injury induced by high glucose through regulating miR-186

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Abstract

Purpose: *To investigate the effects of circ_0001953/miR-186 on human retinal vascular endothelial cell (HRVEC) injury evoked by high glucose.*
Methods: *A cell model (HG group) was established using high glucose-treated HRVECs, while untreated HRVECs were used as the control group (Con group). The levels of endothelin-1 (ET-1), ICAM-1 and IL-6 were evaluated by ELISA and the content of malondialdehyde (MDA) and superoxide dismutase (SOD) in HRVECs were determined. Apoptosis rate was tested adopting flow cytometry. The interrelationship between circ_0001953 and miR-186 was assessed using dual luciferase reporter assay. Measurement of Bax and Bcl-2 was implemented via western blot.*
Results: *In HG group, circ_0001953 increased while miR-186 was downregulated, ET-1, IL-6, ICAM-1, and apoptosis rate increased and accompanied with up-regulated Bax content and declined Bcl-2 protein level. Furthermore, the content of MDA increased and SOD decreased. MiR-186 was a target of circ_0001953.*
Conclusion: *Inhibition of circ_0001953 can repress inflammation, oxidative stress and apoptosis in HRVECs by up-regulating the expression of miR-186.*

Keywords : *circ_0001953, miR-186; HRVECs, inflammation; oxidative stress, apoptosis*

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INTRODUCTION

As a complication that often occurs in patients with diabetes, diabetic retinopathy (DR) can lead to blindness in severe cases [1]. There is no effective treatment for DR, which makes early diagnosis and prevention much important to improve the prognosis of patients [2, 3]. Furthermore, the human retinal vascular endothelial cell (HRVEC) injury is caused by inflammation, oxidative stress and apoptosis, and closely related to DR. Therefore, exploring the pathogenesis of DR and reducing HRVECs damage are of great significance for improving the therapy of DR [4, 5]. Circular RNA (circRNA) plays a key role in new blood vessels formation,

and may be involved in DR occurrence and development though regulating the activity of its target mRNAs though acting as a microRNA (miRNA) sponge [6, 7].

It is known that the expression of circ_0001953 is increased in active tuberculosis, which makes it a possible biomarker for the diagnosis of active tuberculosis [8]. Target gene prediction shows that circ_0001953 has a binding site with miR-186. MiR-186 is down-regulated in osteoarthritis mouse chondrocytes, and contributes to the inhibition of apoptosis via disturbing PI3K-AKT pathway [9]. Nevertheless, the level of circ_0001953 and miR-186 in DR as well as their underlying mechanisms are still unclear. In this study, high glucose-treated HRVECs were used to establish a cell model to unveil the function of circ_0001953/miR-186 in cell injury caused by high glucose.

EXPERIMENTAL

Materials and reagents

Human retinal vascular endothelial cells and DMEM medium were obtained from Gibco (Grand Island, NY, USA); reverse transcription and fluorescent quantitative PCR reagents were purchased from Thermo Fisher (Carlsbad); Trizol® was bought from Invitrogen (Carlsbad, CA, USA); Lipofectamine 2000 and apoptosis detection kit were purchased from Soleibao Technology (Beijing, China); ET-1, IL-6, ICAM-1, SOD and MDA detection kits were bought from Jiancheng Institute of Bioengineering (Nanjing, China); si-NC, si-circ_0001953, miR-NC, miR-186 mimics, anti-miR-NC, and anti-miR-186 were synthesized by Jima Pharmaceutical Technology (Shanghai, Chain); rabbit anti-human primary antibodies and HRP-labeled secondary antibody were provided by Abcam (Cambridge, MA, USA)

Experimental groups

HRVECs were cultured in medium containing 5.5 mmol/L or 30 mmol/L D-glucose for 24 h [10], as control group (Con group) and high glucose treated group (HG group), respectively. When HRVECs were 70% confluent in 24-well plates, transfection was carried out. In brief, an appropriate amount of Lipofectamine 2000 was diluted with 50 μL medium without serum, followed by an appropriate amount of oligonucleotide sequence or vectors diluted with 50 μL medium without serum, and then the two were mixed. The mixture (100 μL) and 400 μL serum-free medium were cultured with HRVECs and 6 h later, the serum-containing medium was utilized. Transfected HRVECs were stimulated with 5.5 mmol/L or 30 mmol/L D-glucose based on the experimental group.

qRT-PCR

Total RNA in HRVECs was gained from cells applying Trizol® in accordance with the guidance of manufacturer, the concentration of purified RNA was detected by an ultraviolet spectrophotometer (HiTaChi, Tokyo, Japan). The solution for reverse transcription PCR was prepared as in the manufacturer's guidelines. At last, qRT-PCR was performed as per the guidelines of the manufacturer [11].

ELISA

Cell supernatant was harvested and the content of ET-1, IL-6 and ICAM-1 of each group was measured using ELISA as per manufactuer's instructions. About 100 μL of each sample and the standard substance were inoculated into the ELISA plate for 90 min incubation. Then 100 μL biotinylated antibodies were added into each well after discarding the liquid, followed by incubation at 37 ℃ for 60 min, adding 100 μL horseradish peroxidase-conjugated working solution to each well for 30 min incubation at 37 ℃. Next, each well was incubated with 90 μL TMB substrate solution away from light and 15 min later, stop solution was utilized. Lastly, the absorbance was measured using a micropore reader.

The detection of SOD and MDA

Cultured cells were collected, and the measurement of the content of SOD and MDA was conducted using their respective commercial kits.

Flow cytometry

In brief, collected cells were digested with 0.25% trypsin and then suspended in PBS solution. About 1×10^6 cells were taken from each sample, and the cells were suspended in 500 μL binding buffer with 5 μL Annexin V-FITC and 5 μL PI (Beyotime, Shanghai, China), consecutively. Apoptosis was detected by flow cytometry.

Dual-luciferase reporter assay

Starbase was employed to analyze the candidate target miRNAs of circ_0001953. The sequences of circ_0001953 3' UTR contained miR-186 binding sites or mutant sequences in binding sites was cloned into pmirGLO plasmid (GenePharma, Shanghai, China). The constructed vectors (named as WT-circ_0001953 or MUT-circ_0001953, respectively) were

transfected into HRVECs in combination with miR-186 mimics or miR-NC. Luciferase activity was evaluated 48 h later, and the activity of renal luciferase was regarded as an internal reference.

Western blot

RIPA lysis (Beyotime) was applied to obtain total protein. Protein denatured at high temperature was used for SDS-PAGE, and the separated proteins were then transferred onto Polyvinylidene Fluoride (PVDF) membranes (Beyotime), and subsequently blocked with 5% skim milk for 2 h. Blocked membranes were incubated with primary antibodies against Bax (ab32503, 1:1000, Abcam), Bcl-2 (ab182858, 1:1000, Abcam), or GAPDH (ab8245, 1:1000) at 4℃ for 24 h, Tris Buffered Saline Tween (TBST) was then used for washing. Next, secondary incubation was conducted with corresponding antibody (ab205718, 1:2000, Abcam) for 1 h at 37 ℃. Eventually, proteins were visualized using an ECL detection kit (Absin, Shanghai, China), and analyzed by Image J.

Statistical analysis

All data were presented as mean ± standard deviation (SD). SPSS statistics 21.0 version was employed for performing statistical analysis. Student's *t*-test or one-way analysis of variance was employed to check the difference between groups. $P < 0.05$ indicated statistically significant.

RESULTS

High glucose promoted the expression of circ_0001953 while inhibited miR-186 level

A cell injury model of DR was established with high glucose. Then relative to the Con group, HRVECs in HG group displayed lower circ_0001953 level and higher miR-186 level (Figure 1).

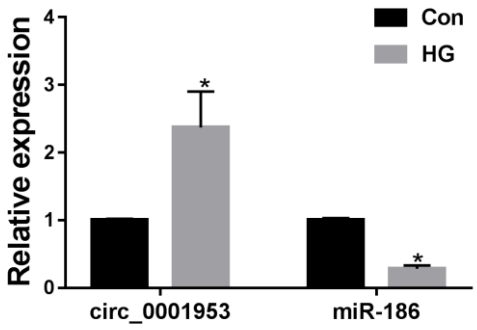

Figure 1: The expression of circ_0001953 and miR-186 in high glucose-treated HRVECs was measured. Relative to Con group, $^{*}P$ <0.05

Circ_0001953 contributed to high glucose induced inflammation and oxidative damage in HRVECs

To probe the contribution of circ_0001953 in cell jury induced by high level of glucose, si-circ_0001953 was transfected into cells. The si-circ_0001953 markedly weakened the expression of circ_0001953 under the exposure of high glucose (Figure 2A). High glucose elevated the levels of ET-1, IL-6 and ICAM-1, which were reversed by circ_0001953 inhibition (Figure 2B). For oxidative damage, results showed increased MDA content and decreased SOD activity in HG group, and these effects were alleviated by si-circ_0001953 (Figure 2C-D). Above results suggested that facilitated high glucose stimulated inflammatory and oxidative damage.

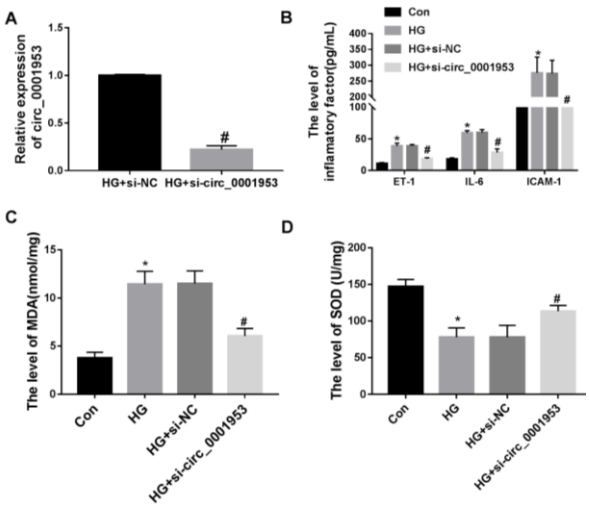

Figure 2: The effect of circ_0001953 inhibition in high glucose-treated HRVECs. (A) Examination of interference efficiency of si-circ_0001953 via qRT-PCR. (B-D) cells were introduced with si-NC or si-circ_0001953, and then cultured with high glucose. (B) ELISA analysis for ET-1, IL-6 and ICAM-1 contents. (C-D) Detection of MDA and SOD levels. $^{*}P$ <0.05, $^{\#}P$ <0.05

Circ_0001953 had functions in high glucose evoked cell apoptosis in HRVECs

As shown in Figure 3A, apoptosis rate, enhanced by high glucose, was blocked after the infection of si-circ_0001953. Furthermore, HG group showed elevated Bax but reduced Bcl-2 in cells, which was reversed by si-circ_0001953 (Figure 3B). Thus, these results suggest that circ_0001953 could promote apoptosis in high glucose-treated HRVECs.

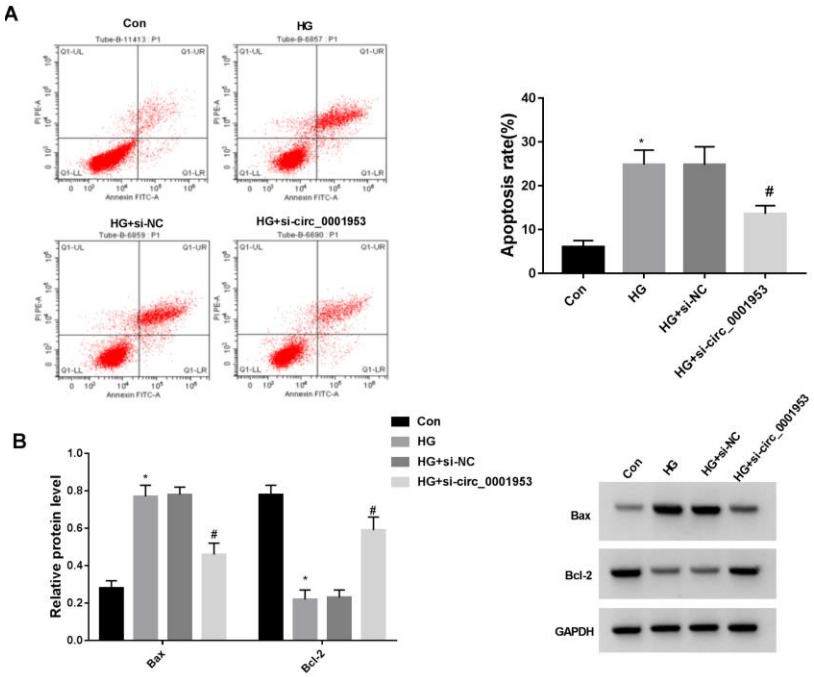

Figure 3: Circ_0001953 could promote apoptosis in high glucose-treated HRVECs. (A) Flow cytometry for apoptosis. (B) Measurement of Bax and Bcl-2 protein levels adopting western blot. $^{*}P$ <0.05, $^{\#}P$ <0.05

MiR-186 is targeted by circ_0001953

CircRNAs can generally serve as sponges of miRNAs. Circular RNA interactome exhibited there are binding sites between miR-186 and circ_0001953 (Figure 4A). To further confirm this connection, dual luciferase reporter assay was employed. MiR-186 overexpression considerably blocked the luciferase activity of WT-circ_0001953 reporter while no obvious change was observed in MUT-circ_0001953 reporter together with overexpressed miR186, suggesting the interrelationship between miR-186 and circ_0001953 (Figure 4B). Consistent with above results, the level of miR-186 was elevated when si-circ_0001953 was introduced (Figure 4C). Therefore, we verified that circ_0001953 suppressed the expression of miR-186.

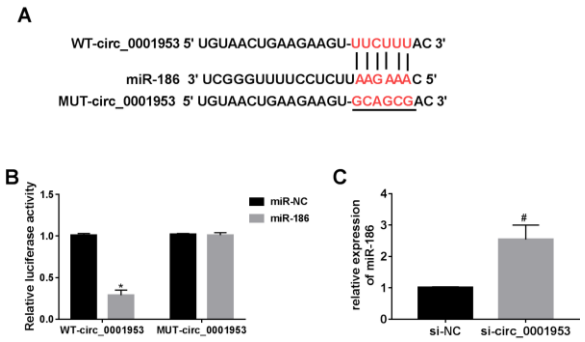

Figure 4: miR-186 is targeted by circ_0001953. (A) Binding sites between circ_0001953 and miR-186. (B) Dual luciferase reporter assay for relationship analysis. (C) Detection of miR-186 via qRT-PCR. $^{*}P$ <0.05, $^{\#}P$ <0.05

MiR-186 inhibited inflammatory and apoptosis while relived oxidative stress in HG-treated HRVECs

For exploring the effect of miR-186 in HG-treated HRVECs, miR-186 mimics was introduced into cells. As expected, Figure 5A showed successful transfection of miR-186 mimics with the elevated miR-186 level in HG+miR-186 group. ELISA assay demonstrated that miR-186 introduction inhibited the content of ET-1, IL-6 and ICAM-1 (Figure 5B). Moreover, oxidative stress was relieved with additional miR-186 (Figure 5C-D). It was also observed that the apoptosis rate of HG-treated HRVECs was lowered as manifested by high-expressed Bcl-2 and low-expressed Bax (Figure 5E-F). Altogether, miR-186 inhibited inflammatory and apoptosis while relieved oxidative stress in HG-treated HRVECs.

MiR-186 absent reversed the effect caused by si-circ_0001953 in HG-treated HRVECs

To directly unveil whether miR-186 was a functional target for circ_0001953, si-circ_0001953 and/or anti-miR-186 was co-transfected into HRVECs. As described in Figure 6A, the expression of miR-186 decreased in HG+si-circ_0001953+anti-miR-186 group. In addition, the level of inflammatory factors, MDA, Bax as well as apoptosis rate were elevated while SOD and Bcl-2 decreased (Figured 6B-F). In all, anti-miR-186 abated the functions caused by si-circ_0001953 in HG-treated HRVECs.

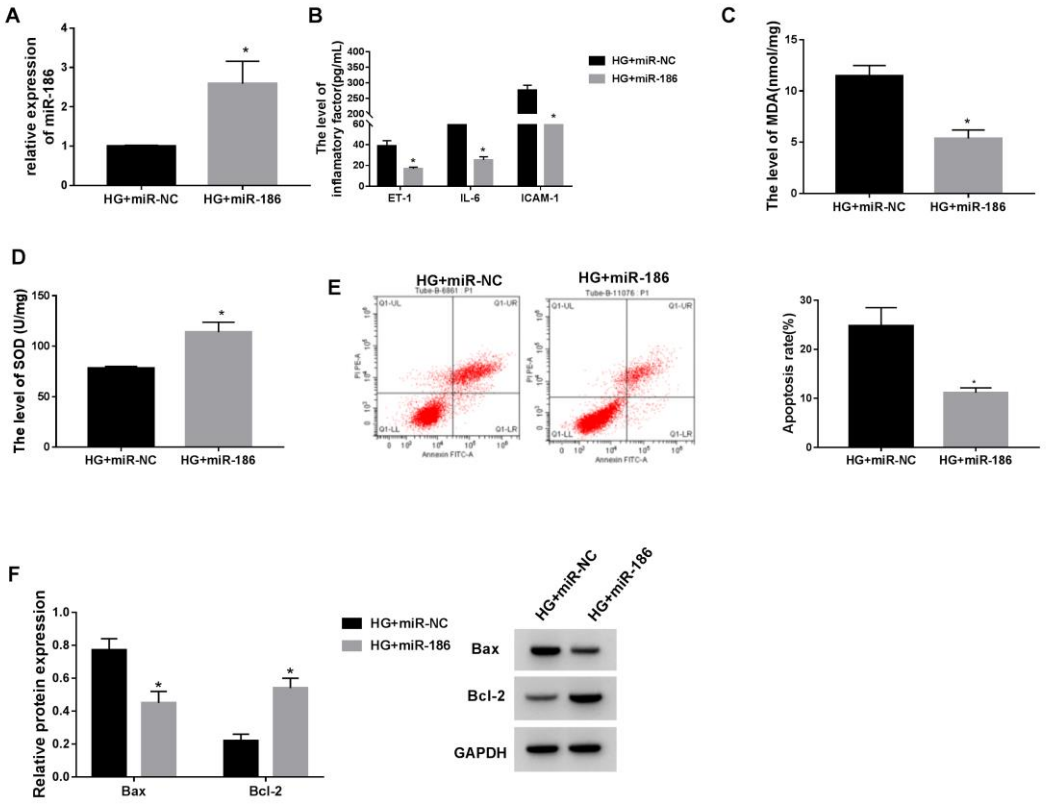

Figure 5: miR-186 inhibited inflammatory and apoptosis but relieved oxidative stress. (A-F) miR-NC and miR-186 were infected into cells. (A) Measurement of miR-186 via qRT-PCR. (B) The level of inflammatory factors evaluated by ELISA. (C, D) Measurement of MDA and SOD contents. (E) Flow cytometry for apoptosis rate detection. (F) Western blot for Bax and Bcl-2 levels. $^{*}P$ <0.05

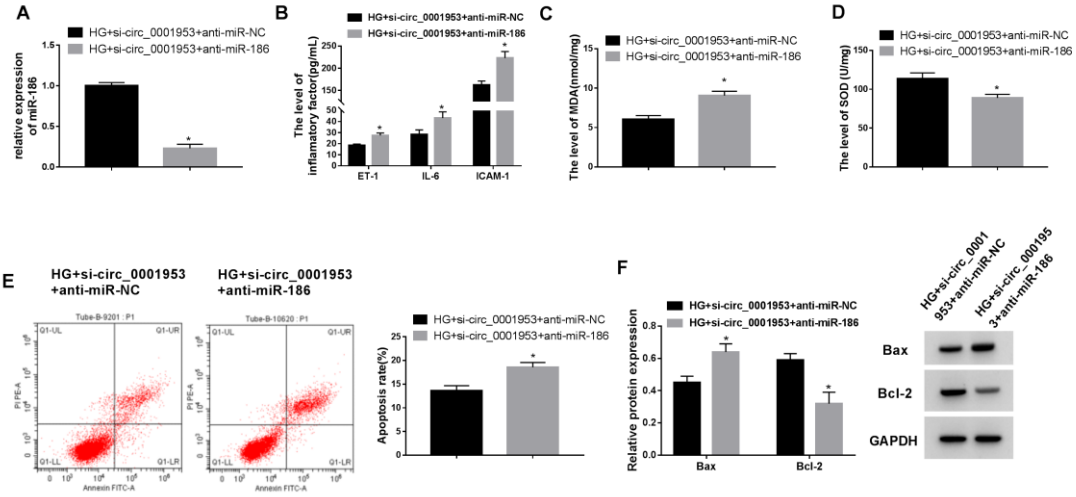

Figure 6: Effect caused by si-circ_0001953 was blocked by anti-miR-186. (A-F) si-circ_0001953 and anti-NC or anti-miR-186 transfected HRVECs were treated with high glucose. (A) Measurement of miR-186 utilizing qRT-PCR. (B) Levels of inflammatory factors was evaluated by ELISA. (C, D) Measurement of MDA and SOD contents. (E) Analysis of apoptosis rate via flow cytometry. (F) Western blot for Bax and Bcl-2 levels. $^{*}P$ <0.05

DISCUSSION

Inflammation, oxidative stress and other reactions are intensified in high glucose condition, which leads to apoptosis of HRVECs, and further promotes the development of DR [5, 12]. CircRNA-ZNF532 was proved to regulate the periretinal cell degeneration and vascular

dysfunction caused by diabetes [13]. CircRNA COL1A2 boosts the angiogenesis of DR by modulating miR-29b/VEGF axis [14]. CircRNA_0084043 enhanced the inflammatory and oxidative damage triggered by HG in retinal pigment epithelial cells via elevating TGFA through sequestering miR-140-3p [6]. Circ_0001953 is reported to be up-regulated in proliferative diabetic retinopathy, which makes it a potential diagnosis biomarker [15, 16]. However, the mechanism of circ_0001953 in the development of DR has not been fully studied.

In this study, high glucose exposure exhibited higher circ_0001953 expression in HRVECs, suggesting circ_0001953 may play a role in DR development. Consistent with previous studies, our data manifested the increased levels of ET-1, IL-6, ICAM-1 and MDA after high glucose treatment, while SOD level decreased [17], indicated that high glucose aggravated the damage of HRVECs through promoting inflammation and oxidative stress. Further analysis proved the interference of circ_0001953 could partially inhibit high glucose-triggered inflammation and oxidative stress and thus reduced cell damage in HRVECs. In line with previous findings [18], high glucose level evoked apoptosis, which was also characterized as the enrichment of Bax protein and reduced level of Bcl-2 protein. Moreover, this effect was blocked after the inhibition of circ_0001953.

MiRNAs play crucial roles in the regulation of gene expression [19, 20]. The effects of miR-186 in various diseases have drown more attention. Dang *et al.* have shown that hsa_circ_0010729 affects the survival of vascular ECs via miR-186/HIF-1alpha axis [21]. Chen *et al.* proved that miR-186 inhibited neuropathic pain by regulating NLRP3 signaling pathway [22]. Lowly expressed miR-186-5p contributes to cardiomyocyte apoptosis evoked by HG by targeting TLR 3 [23]. MiR-186-5p Inhibition can lead to cardiomyocyte damage induced by high glucose [24]. MiR-186 was considered to be involved in chronic obstructive pulmonary pathogenesis through targeting HIF-1α [25]. Herein, functional experiments showed that miR-186 overexpression arrested inflammatory and oxidative damage in HG-challenged HRVECs. In addition, the reduction of miR-186 in high glucose-treated HRVECs was observed. Meanwhile, miR-186 deficiency abolished the functions of circ_0001953 interference on inflammatory, oxidative damage and apoptosis, suggesting circ_0001953 could function as miR-186 sponge in DR development.

CONCLUSION

This study has demonstrated that circ_0001953 increased in high glucose-challenged HRVECs, while the expression of miR-186 was decreased. Circ_0001953 insufficiency inhibited cell inflammation, oxidative stress and apoptosis to alleviate cell damage by up-regulating miR-186. The circ_0001953/miR-186 axis may be responsible for DR progression, and benefited for developing new strategy for DR prevention.

DECLARATIONS

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Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We 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 the authors.

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