Letter to the Editor

Dynamic Expression of Hyperpolarization-activated Cyclic Nucleotide-gated Cation Channel 4 Involved in Microwave Induced Pacemaker Cell Injuries

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To investigate the mechanisms of microwave induced pacemaker cell injuries, Wistar rats and the primary pacemaker cells of newborn Wistar rats were exposed to microwave at average power density of 50 mW/cm^2. Slower spontaneous beating rate, intercellular Ca^{2+} aggregation and cell membrane perforation were detected immediately after the exposure. Moreover, hyperpolarization-activated cyclic nucleotide-gated cation channel 4 (HCN4) was down-regulated immediately after the exposure and up-regulated at 12 h after the exposure. In the sinoatrial node (SAN) of the rats, HCN4 expression increased from day 1 to day 28 after microwave exposure, then declined from 3rd month to 6th month, which was consistent with the changes of ratio of β1-adrenergic receptor (β1-AR) and muscarinic type 2 acetylcholine receptor (M2-AchR). In conclusion, dynamic expression of HCN4, together with changes of β1-AR and M2-AchR expression, was involved in microwave induced pacemaker cell injuries.

In persons with long-term exposure to microwave, the impairment of cardiac function, such as sinus tachycardia, sinus arrhythmia and atrioventricular block, could be detected[1]. In animal models, microwave could cause structural, functional damages of sinoatrial node (SAN) and the crucial component of cardiac conduction system[2-3]. Pacemaker cells are clustered in SAN and generate action potentials (APs) to maintain normal heart rhythm. At the end of a sinoatrial APs, the funny current (I_f) flowing through hyperpolarization-activated cyclic nucleotide-gated cation channels (HCNs) on pacemaker cells is activated and supplies inward current to control the cardiac rate. HCN4 is the main isoform of HCNs in SAN, and its mutation or changes of expression levels was responsible for different forms of heart diseases, such as arrhythmia, sinus bradycardia, ischemia-reperfusion induced injury, long QT interval, polymorphic ventricular tachycardia, and so on[4-5]. In this study, we investigated the roles and mechanisms of HCN_4 in microwave induced injuries of pacemaker cells.

The microwave source equipment and exposure procedures have been described previously[3]. All the procedures for experimental animals were approved by the Ethics Committee in Beijing Institute of Radiation Medicine.

Sixty male adult Wistar rats (200±20 g) were divided randomly into a 50 mW/cm^2 microwave exposure (MW) group and a sham-exposure (SH) group. The rats in the MW group were exposed to microwave radiation at average power density of 50 mW/cm^2 for 6 min. The rats in the SH group were treated without microwave radiation under the same conditions. On day 1, day 7, day 14, day 28 and at 3rd month and 6th month after microwave exposure, 5 rats from each group were sacrificed and the tissues containing SAN were obtained. The mRNA expression of HCN4 was detected by in situ hybridization (ISH) using HCN4 probe (MK3601-r, Boster Biological Technology Ltd, Wuhan, China) according to the manufacturer's instructions. The protein expression of HCN4, β1-AR and M2-AchR were assayed by using mouse anti-rat HCN4 antibody (Abcam, Cambridge, MA, United Kingdom), rabbit anti-rat β1-AR antibody (Biosynthesis Biotechnology Co.Ltd, Beijing, China) and rabbit anti-rat M2-AchR antibody (Boster Biological Technology Ltd), respectively, according to the standard protocol of immunohistochemistry (IHC). The integral optical density (IOD) of mRNA and protein expression was quantified from three random 200× images by using CMIAS-II image analysis system (BeihangMotic Inc., Beijing, China).

Pacemaker cells were isolated from newborn

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Wistar rats as reported previously[6]. The cells were cultured in dulbecco's minimal essential medium (DMEM) (Neuronbc, Shenzhen, China) plus 15% newborn calf serum (NCS, Sigma-Aldrich Co.) at density of (1-10)×10^5/mL. And, 0.1 mmol/L 5-bromo-2- deoxyuridine (5-BrdU, Sigma-Aldrich Co.) was added at 72nd h to inhibit the growth of fibroblasts. The morphological characteristics and spontaneous beating rates were monitored at 12th h, 24th h, 48th h, and 72nd h.

After 5 d *in vitro* culture, the primary pacemaker cells were exposed to 50 mW/cm^2 microwave. Before the exposure, immediately and 12 h after the exposure, the expressions of HCN4 were detected with immunofluorescence staining by using mouse anti-rat HCN4 antibody (Abcam), and observed with laser scanning confocal microscopy (LSCM, Carl Zeiss, Oberkochen, Germany). Moreover, immediately after the exposure, the intercellular Ca^{2+} were labeled with the calcium fluorescent probe, Fluo-3-AM (Molecular Probes, Eugene, OR, USA), and analyzed with LSCM. The fluorescence intensity of Ca^{2+} in 10 random cells was analyzed with Volocity Analysis software. Furthermore, the pacemaker cells were fixed by using 3% glutaraldehyde immediately after the exposure to detect the structure of cellular membrane with NanoWizard BioScience atomic force microscope (AFM) system (JPK Instruments, Berlin, Germany).

All the data presented were based on the results of at least three independent experiments and analyzed with statistical software SPSS 17.0 (Chicago, USA). All the values were shown as mean±SD. One-way ANOVA and Student's *t*-test were applied for statistical analysis.

In our previous studies, we have demonstrated that 50 mW/cm^2 microwave exposure could cause sinus arrhythmia, atrial arrhythmias and ultra-structural injuries of SAN in rats[3]. In this study, we focused on the effects of microwave exposure on pacemaker cells, the specialized cells in SAN generating rhythmic excitement and controling cardiac rate. Pacemaker cells were spindle-shaped cells with the fastest spontaneous beating rate (152±16 times/min) and high level of HCN4 expression. Immediately after the exposure, slower spontaneous beating rate (84.4±16.7 times/min), and reduced pseudopods and protrusions formation could be observed. Although the spontaneous beating rate restored to 146.8±24.2 times/min in most of the healthy pacemaker cells, significant cell death and irregular beating rhythm could be still detected at 12 h after the exposure (Figure 1A-1C).

The concentration and distribution of the intercellular Ca^{2+} play pivotal roles to maintain cardiac rhythm. And, intercellular Ca^{2+} overloading was responsible for some pathological injuries of heart, while Ca^{2+} channel blockers could prevent the damage obviously[7]. In this study, we found that microwave exposure induced the dramatic increase of intercellular Ca^{2+} immediately after the exposure (Figure 1D). Moreover, the results of AFM showed that microwave exposure increased the membrane folds and produced more visible pores with different sizes on the membranes, suggesting that membrane perforation had been induced (Figure 1E). These data indicated that the intercellular Ca^{2+} aggregation was involved in microwave induced pacemaker cells injuries, and might be caused by the changes of cell membrane permeability. Taken together with previous reports from our group and others, cell membrane perforation, impairment of Ca^{2+}-Mg^{2+}-ATPase activities, mitochondrial injury and reactive oxygen radicals (ROS) production are possible underlying mechanisms of intercellular Ca^{2+} aggregation, which augments microwave induced cell injuries and leads to cell death[8].

HCN4, the main inform of HCNs expressed in SAN, mediated I_f to generate rhythmic firing (automaticity) in pacemaker cells and sustain heart rhythm. In the primary pacemaker cells, a slight decrease of HCN_4 expression was detected immediately after microwave exposure, and then was significantly up-regulated at 12 h after the exposure (Figure 2A-2B), suggesting that certain mechanisms was induced to stimulate HCNs expression. Moreover, we also detected the HCN4 expression in the SAN of the rats. Our data showed that HCN4 mRNA expression was induced rapidly by microwave exposure and reached the highest level on day 1 after the exposure, and then gradually decreased over time (Figure 2C, 2E). In addition, HCN4 protein expression began to increase at day 1 after the exposure and peaked on day 14, and then decreased from day 14 to 6th m (Figure 2D, 2F). Compared with the SH group, both the mRNA expression and protein expression of HCN4 were up-regulated from day 1 to day 28, and down-regulated from 3rd month to 6th month after the exposure in the MW group.

Several studies showed that the disorders of sympathetic and parasympathetic nervous system was the major cause for microwave induced cardiovascular injuries[9]. β1-AR and M2-AchR are two

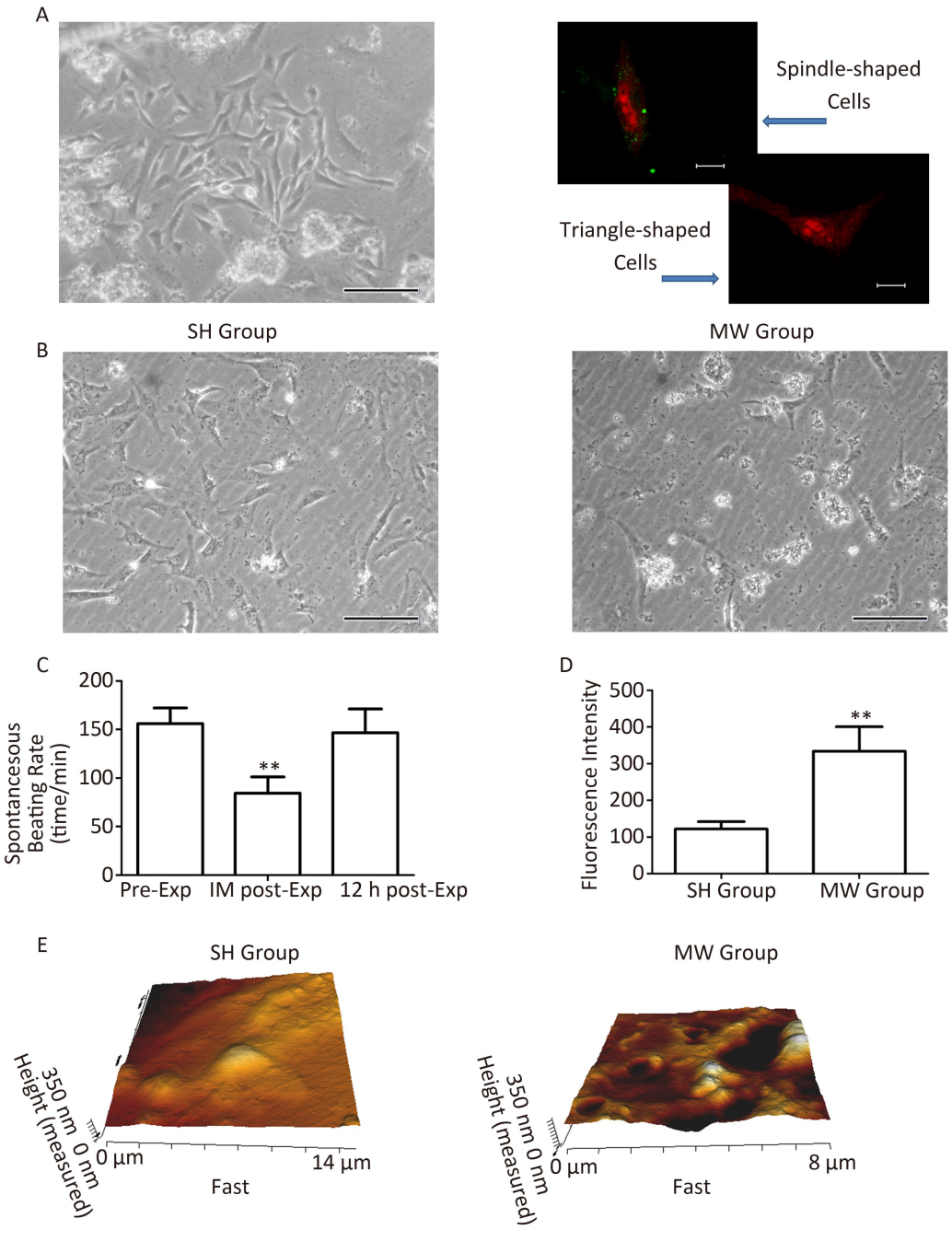

Figure 1. Microwave induced morphological and functional damages on pacemaker cells. Primary pacemaker cells were isolated from newborn Wistar rats and cultured *in vitro* for 5 d. HCN4 were detected with LSCM. The Spindle-shaped and HCN4 expressed cells were identified as pacemaker cells (A). Twelve hours after the exposure to 50 mW/cm^2 microwave, the morphological changes of the pacemaker cells (B) were observed. And, the spontaneous beating rates (C) were detected before the exposure, immediately and 12 h after the exposure. Moreover, intercellular Ca^{2+} was labeled with calcium fluorescent probe, Fluo-3-AM and observed with LSCM immediately after the exposure. 10 pacemaker cells in each group were selected to obtain average fluorescence intensity (D). Structures of cell membrane were also detected with AFM immediately after the exposure, and the representative images were shown in E. Scale bar represents 10 μm in the right panel of A, and represents 50 μm in other images. Data were shown as mean±SD. $^{**}P<0.01$ *vs.* SH group.

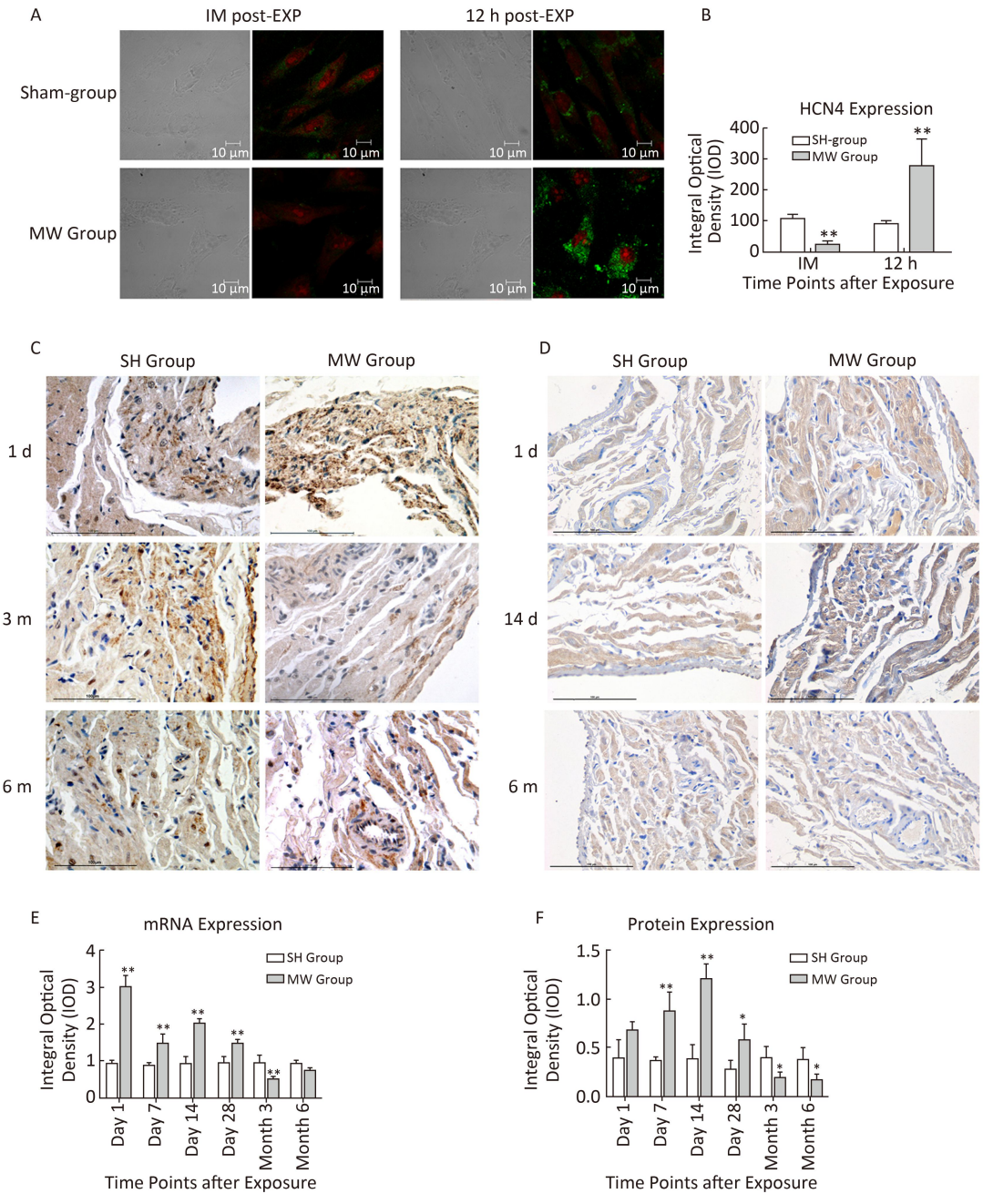

Figure 2. Microwave regulated HCN4 expression in pacemaker cells and rats' SAN. Primary pacemaker cells were exposed to 50 mW/cm^2 microwave, and the expression of HCN4 was detected by using immunofluorescence staining immediately and 12 h after the exposure by using LSCM (A). The fluorescence intensities of HCN4 staining were analyzed by using Volocity Analysis software and the average intensity was obtained from ten randomly selected pacemaker cells (B). Wistar rats were exposed to 50 mW/cm^2 microwave, and the mRNA and protein expression of HCN4 in rats' SAN were detected with ISH and IHC on day 1, day 7, day 14, day 28 and at 3rd month and 6th month after the exposure. The representative images were shown in (scale bar=100 μm). The integral optical density (IOD) was quantified from three random 200 × images by CMIAS-II image analysis system.The data in B, E and F was shown as mean±SD. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* SH group at the same time point after microwave exposure.

major receptors for noradrenaline and acetylcholine respectively, and the imbalance of β1-AR and M2-AchR is closely associated with several cardiovascular diseases[10]. In this study, we found that both β1-AR and M2-AchR expression could be induced by microwave exposure from day 1 to 3rd m after the exposure. However, the β1-AR expression peaked on day 7 after the exposure, while M2-AchR expression peaked at 3rd month after the exposure (Figure 3A-3D). It has been reported that β1-AR and M2-AchR played converse roles in regulating the activities of adenylatecyclase (AC) and producing cyclic adenosine monophosphate (cAMP), which could bind and activate HCN4 ion channel. Thus, we analyzed the changes of the ratio of β1-AR and M2-AchR after microwave exposure. Interestingly, the ratio of β1-AR and M2-AchR expression increased from day 1 to day 28 and then decreased from day 28

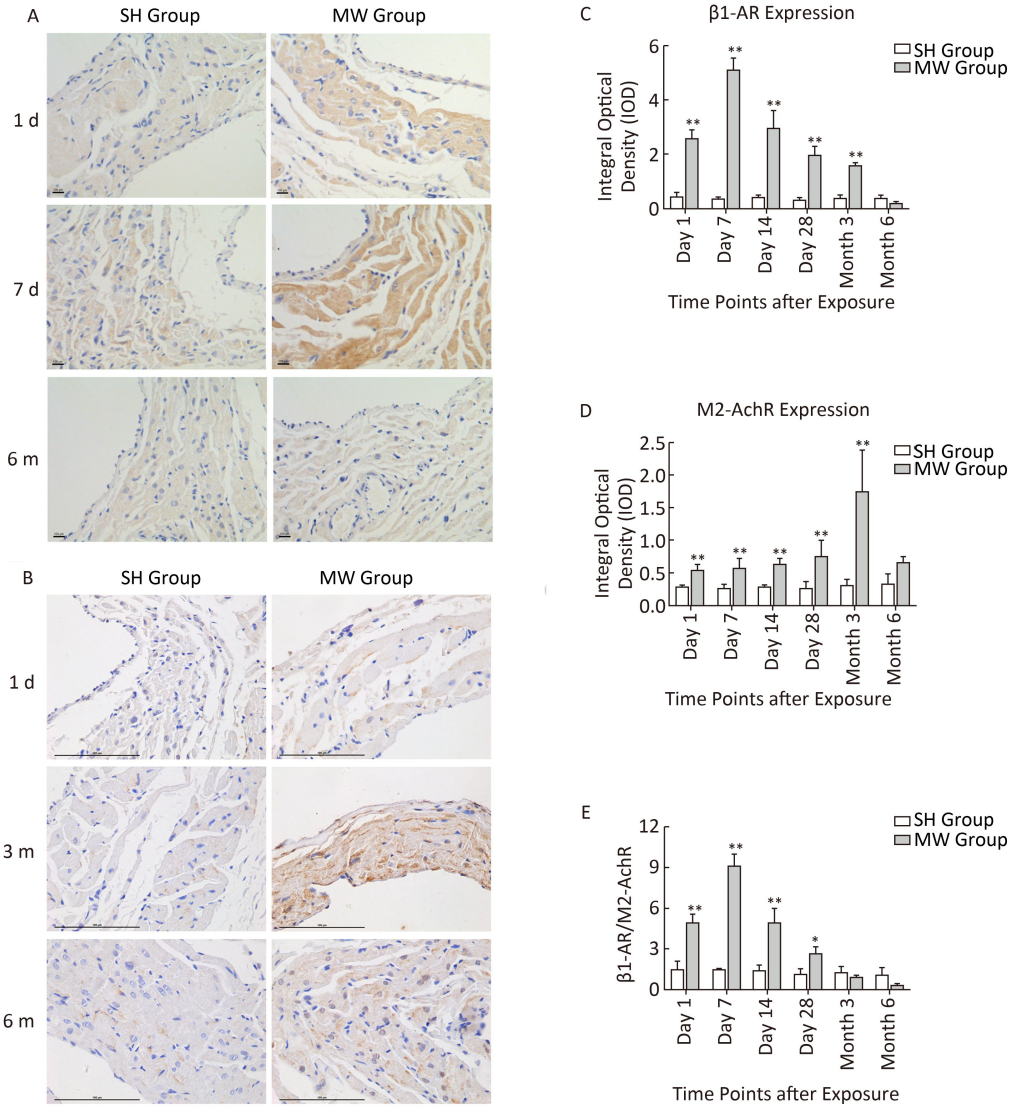

Figure 3. Microwave induced β1-AR and M2-AchR expression in rats' SAN. Wistar rats were exposed to 50 mW/cm^2 microwave as described in the materials and methods. On day 1, day 7, day 14, day 28 and at 3rd month and 6th month after microwave exposure, rats in MW group and SH group were sacrificed and the tissues containing SAN were collected. Protein expression of β1-AR and M2-AchR were detected by IHC. The representative images were shown in A and B (scale bar=100 μm). The IOD of β1-AR and M2-AchR were quantified from three random 200× images by using CMIAS-II image analysis system (C and D). The ratio of β1-AR and M2-AchR expression was calculated and presented in E. The data in C, D, and E was shown as mean±SD. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* SH group at the same time point.

to 6th month, which was consistent with the HCN4 expression after microwave exposure, suggesting that β1-AR and M2-AchR might be involved in microwave mediated regulation of HCN4 expression (Figure 3E).

In conclusion, 50 mW/cm^2 microwave exposure could induce pacemaker cell injuries probably through a β1-AR and M2-AchR mediated regulatory mechanism of HCN4 expression. Moreover, microwave exposure induced intercellular Ca^{2+} aggregation and mitochondria injury might aggravate the damages of pacemaker cells and result in cell death.

Competing Interests The author(s) declare that they have no competing interests.

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