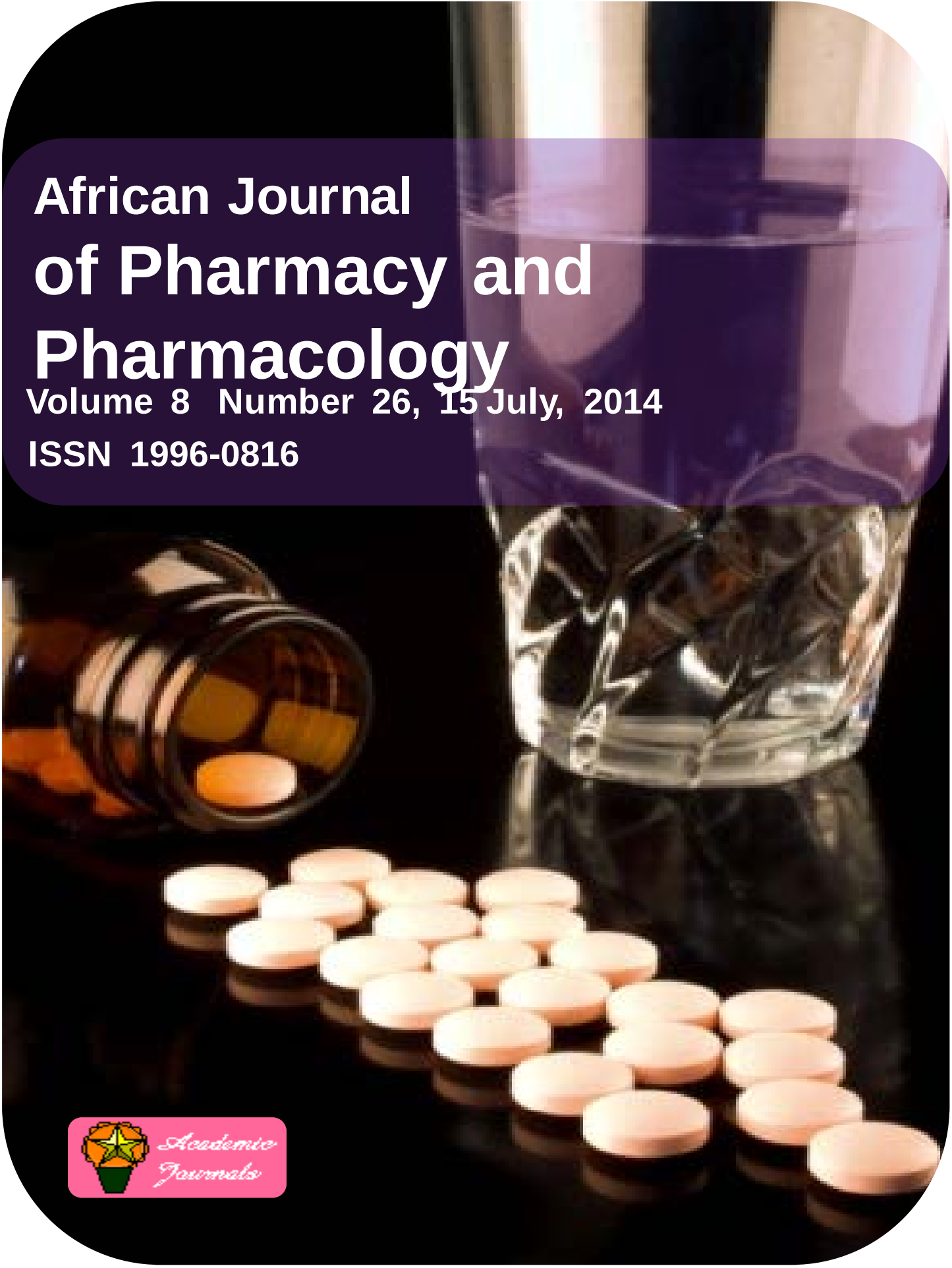

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Full Length Research Paper

Nitric oxide protects rat cultured cardiomyocytes from apoptosis in apparent association with decreased Ca^{2+}/calmodulin-dependent protein kinase II activity

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Nitric oxide (NO) is an important signaling molecule involved in various processes such as cardiac ischemia/reperfusion (I/R) injury and diabetes. In heart, numerous studies have reported that NO can promote or inhibit apoptosis. However, to date, the exact mechanism of the protective role of NO in cardiac apoptosis is still obscure. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a serine/threonine-specific protein kinase whose expression and activity is up-regulated in cardiac ischemia and hypertrophy. In this study, we evaluated the effect and CaMKII-related mechanism of NO on cardiomyocyte apoptosis using electric field stimulation (EFS) and *in vitro* I/R as the apoptotic stimulator. We found that S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP), a donor of NO, significantly reduced the increase in lactate dehydrogenase (LDH) content, apoptotic nuclei number and cleaved caspase-3 level in EFS and I/R-stimulated cardiomyocytes. The effect of SNAP was found to be mediated by the reduction in the expression of Bax protein and mRNA in EFS- or I/R-stimulated cardiomyocytes. Unlike Bax, the level of Bcl-2 protein and mRNA was not changed by SNAP. Further mechanistic studies showed that SNAP treatment reduced the increase in the phosphorylation level of phospholamban (PLB) in EFS- or I/R-stimulated cardiomyocytes, and this reduction might mediate the formation of apoptosis because KN93, a specifiec inhibitor of CaMKII, reduced the increase in the expression of Bax protein in I/R-stimulated cardiomyocytes. Taken together, our results indicate that NO protects rat cultured cardiomyocytes from apoptosis likely by suppressing the activity of CaMKII and the subsequent reduction of Bax expression.

Key words: Nitric oxide (NO), Ca^{2+}-calmodulin dependent kinase II (CaMKII), ischemia/reperfusion (I/R), electric field stimulation (EFS), cardiomyocyte.

INTRODUCTION

Nitric oxide (NO), a gas molecule found in 1992 (Miller et al., 2007), is an important mediator of various physiological and pathological processes, such as vascular tone regulation, leukocyte-endothelium adherence, platelet

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platelet aggregation, and ischemia/ reperfusion (I/R) damage (Ohmori et al., 1998). In the body, the effects of NO depend on the surrounding environment. When tissues or cells are exposed to excessive NO, toxic effects will occur. Protective effects have been presented in numerous cases. For example, it has been shown that application of NO donor during an ischemic insult limits the reperfusion damage to heart (Bolli, 2001), liver (Aiba et al., 2001), and brain (Sato et al., 1994). Based on the opposite effects of NO, there occurs a lot of debates concerning whether NO is detrimental or protective among scientists. In fact, the majority of literatures from the past 15 years demonstrate that NO is indeed protective, at least in the process of cardiac ischemia/reperfusion (I/R) (Gourine et al., 2002; Kawahara et al., 2006; Hotta et al., 1999). NO was also found to exhibit protective function in apoptosis in other cells. For instance, NO donor was reported to protect vascular cells (Duran et al., 2009) and human extravillous trophoblast cells (Dash et al., 2003) from apoptosis via preserving p53 levels or elevating cGMP levels, respectively.

In the present study, we employed two apoptosis-induced stimulation electric field stimulation (EFS) (Liu et al., 2010) and *in vitro* I/R (Snoeckx et al., 1990) to evaluate the protective role of NO in cardiomyocyte apoptosis. We found that EFS and I/R stimulation induces apoptosis in primary cardiomyocytes and NO attenuated the occurrence of apoptosis by decreasing the expression of Bax protein.

Although plenty of evidence for the protective role of NO in I/R-mediated damage in cardiomyocytes have been reported in the past years, the exact mechanism is still unclear, especially for the role of NO in EFS-induced apoptosis. Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is a holo-enzyme that can mediate a lot of processes. Over-expression of CaMKIIδB and CaMKIIδC (Anderson et al., 2011; Schott et al., 2010; Chiba et al., 2008) exacerbates myocardial hypertrophy and heart failure. The CaMKII pathway is considered as a core mechanism for promoting cardiac hypertrophy and heart failure, which is tightly associated with cardiac apoptosis. It has also been reported that overexpression of CaMKIIδC promotes cardiomyocyte apoptosis (Zhu et al., 2007) and inhibition of CaMKII protects cardiomyocytes from necrosis and apoptosis in irreversible I/R injury (Vila-Petroff et al., 2006). In addition, it is worth noting that CaMKII can be inactivated by NO-mediated S-nitrosylation at cysteine (Takata et al., 2011; Song et al., 2008). Thus we speculate that NO might exert anti-apoptotic role via attenuating CaMKII activity in primary cardiomyocytes. In fact, we found that S-Nitroso-N-Acetyl-D,L-Penicillamine (SNAP) reduced the increase in the phosphorylation level of phosphorylation level of phospholamban (PLB), a downstream target of CaMKII, in EFS- or I/R-stimulated cardiomyocytes, and inhibition of CaMKII decreased the expression of Bax protein. Taken together, our results indicate that the protective role of NO in cardiomyocyte apoptosis is apparently associated with the alteration of Bax expression and CaMKII activity.

MATERIALS AND METHODS

Dulbecco's modified eagle's medium/F12 (DMEM/F12) was the product of Gibco Invitrogen Corporation (Carlsbad, CA, USA). Fetal bovine serum (FBS) was purchased from Hyclone Corporation (Logan, UT, USA). 1% penicillin-streptomycin KN93, SNAP, and Hoechst 33258 were purchased from Sigma (Saint Louis, MO, USA). TC was purchased from Tocris Bioscience (Ellisville, MO, USA). Antibodies against Bax, Bcl-2, PLB, phospho-PLB, caspase-3, and β-actin were the products of Cell Signaling Technology (Beverly, MA, USA). IRDye 680 donkey anti-rabbit IgG and anti-mouse IgG secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE, USA). Other related agents were purchased from commercial suppliers. SNAP and KN93 were dissolved in dimethylsulfoxide (DMSO). The final concentration of DMSO was < 0.05%. No detectable effects of DMSO were found in our experiments. All drugs were prepared as stock solutions, and stock solutions were stored at –20°C.

Cell preparation

Ventricular cardiomyocytes were isolated from neonatal rats (day 0 to 1). Briefly, ventricles were excised and washed three times with PBS containing 136.8 mM NaCl, 2.6 mM KCl, 0.88 mM KH_2PO_4, 9.7 mM Na_2HPO_4, and 1% PS, pH 7.4, to wash blood clots out. After that, clean ventricles were dissected carefully into small shivers using a special scissor. Shivers were then transferred into a phialis to digest with 0.6 mg/ml trypsin PBS solution (37°C, 20 min). Next, the supernatants were discarded after centrifugation (118 *g,* 5 min) and the precipitates were re-suspended in 10% heat-inactivated fetal bovine serum (FBS). The steps were repeated 7 to 10 times until the ventricles were completely digested. Then, cell suspensions were placed in culture dish in humidified 5% CO_2/95% air at 37°C. After 1.5 h, the supernatants were removed and diluted to the confluence 1×10^6/ml and placed in six well tissue culture dishes with DMEM/F12 containing 10% FBS and 1% penicillin-streptomycin (100 U/ml). Bromodeoxyuridine (0.1 mM) (Sigma, Saint Louis, MO, USA) was added to prevent the proliferation of non-cardiomyocytes. All cells were kept in humidified 5% CO_2/95% air at 37°C. The purity of cardiomyocytes is > 95%.

Cell viability assay

Cell viability was measured using MTT assay according to the manufacturer's protocol (Bi Yuntian Biological Technology Institution, Haimen, China). Briefly, cultured cells in plates were washed with PBS and then exposed to MTT solutions (5 mg/ml) and kept at 37°C for 4 h. The blue crystals were dissolved in stop solution containing DMSO and the absorbance was read at 490 nm.

LDH determination

The injury of cardiomyocytes was assessed by measuring the content of lactate dehydrogenase (LDH) in the supernatants. The enzyme was determined by using an assay kit according to the manufacturer's protocol (Bi Yuntian Biological Technology Institution,

Haimen, China). The absorbance of the samples was read at 440 nm.

Hoechst 33258 staining

For Hoechst 33258 staining, cells in different groups were fixed and treated with Hoechst 33258 staining solution, mixed gently and stained for 20 to 30 min at 4°C, then observed cells using fluorescent microscope. Cells with condensed bright nucleus were regarded as apoptotic cells. The apoptosis rate was calculated by the ratio between the numbers of cells with condensed bright nucleus and total cell numbers. The number of apoptotic cells was counted from the resulting four phases for each point with a digital camera and microscope (Olympus, Japan), and then averaged for each experimental condition. The data presented were generated from three separate assays.

In vitro I/R model

Lack of oxygen and glucose was used to mimic *in vitro* ischemia and recovering to normal growth conditions was used to mimic reperfusion. The growth medium was replaced with D-Hanks buffers containing 137 mM NaCl, 5.4 mM KCl, 0.37 mM Na_2HPO_4, 0.44 mM KH_2PO_4 and 4.2 mM $NaHCO_3$, pH 7.4, and the cells were transferred to a hypoxic incubator (Thermo, USA) filled with saturated nitrogen to incubate 4 h. Oxygen was adjusted to 1.0% and CO_2 to 5.0%. Re-oxygenation was initiated by replacing the hypoxia solutions with D-Hanks buffer including Ca^{2+}, Mg^{2+} and glucose. The cells were then cultured in the incubator under an atmosphere of 5% (v/v) CO_2 in air at 37°C for further 12 h. Previous time-course studies showed that 4 h hypoxia and 12 h re-oxygenation were enough to induce cell death. No treated cells were as controls.

Electrical field stimulation

Cells were stimulated with L-channel C-Pace chronic stimulation unit purchased from Ion-Optics (Milton, MA, USA). The pulse of 10 V, 3 Hz and 4 ms was employed to induce cell apoptosis. After 5 min stimulation, cells went on incubation for 3 h for further analysis.

Reverse transcription-polymerase chain reaction (RT-PCR)

For RT-PCR, total RNA was isolated from cultured cardiomyocytes using Trizol reagent (Invitrogen) followed by isopropanol precipitation. RNA concentration was assessed by spectrophotometry and 2 µg RNA was used for cDNA synthesis with the RevertAidTM First Strand cDNA Synthesis system for RT-PCR kits (Fermentas). Primers for Bcl-2, Bax and GAPDH were as follows: Bcl-2, 5'-CCGGGTGGCAGCTGACATGTTT-3' (forward), 5'-GCACAGGGCCTTGAGCACCAGTT-3' (reverse); Bax, 5'-CGGGCCCAC CAGCTCTGAACA-3' (forward), 5'-GGGCGGCTGCTCCAAGGTCA-3' (reverse); GAPDH, 5'-CCATGTTCGTCATGGGTGTGAACCA-3' (forward), 5'-GCCAGTAGAGGCAGGGATGATGTTC-3' (reverse). Three micro-liters of the first strand product were used as a template in each 25 µl PCR reactions. The cycling parameters consisted of an initial step of 5 min at 95°C followed by 33 cycles of 45 s at 95°C, 45 s at 56°C, 1 min at 72°C, and a terminal process of 7 min at 72°C. For standardization of individual RT-PCR reactions, GAPDH-specific amplifications were performed in parallel. The PCR reaction products were analyzed by agarose gel electrophoresis and scanning densitometry. The data were normalized by the GAPDH in the same group.

Western blot

To extract the total proteins, cultured ventricular cardiomyocytes were lysed on ice for 30 min in lyses buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 100 mM NaCl, 20 mM NaF, 3 mM Na_3VO_4, 1 mM PMSF, with 1% Nonidet P-40, and protease inhibitor cocktail). The lysates were centrifuged at 12000 *g* for 15 min, and the supernatants were recovered. After denatured, 30 µg of proteins were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) gels and then transferred to nitrocellulose membranes by using a transfer cell system (Bio-Rad, California, USA). After blocking with 5% nonfat dried milk powder/Tris-buffered saline Tween-20 (TBST) for 1 h, membranes were probed with 1:500 primary antibodies against caspase-3, Bcl-2, Bax, PLB, phospho-PLB or 1:5000 primary antibody against β-actin overnight at 4°C. Primary antibodies were then removed by washing the membranes 3 times in TBST, and incubated for further 1 h at room temperature with IRDye 680-labled secondary antibodies (1:3000 to 1:5000). Immunoblots were visualized by scanning using Odyssey CLx western blot detection system. The band density was quantified using Image J software. All assays were performed at least three times.

Statistical analysis

Data are expressed as means ± standard error (SE). One-way analysis of variance (ANOVA) followed by post hoc test was used for the statistical analysis by employing statistical package for social sciences (SPSS) 11.0 software. Differences were considered statistically significant at $P < 0.05$ or $P < 0.01$.

RESULTS

NO inhibits the apoptosis of primary cardiomyocytes induced by EFS

EFS is a stimulation widely used to interfere with the function of cells (Duran et al., 2009; Dash et al., 2003). In this study, we used EFS to stimulate cardiomyocytes. We found that EFS (10 V, 3 Hz, 4 ms pulses) significantly decreased the survival rate of primary cardiomyocytes (Figure 1A). Pretreatment of cardiomyocytes with SNAP (30 min), a donor of NO, partially reversed the decrease in the survival rate of EFS-stimulated cardiomyocytes, and this reversion was statistically significant at the concentration of 1 mM but not at 2 mM (Figure 1A). For this reason, 30 min and 1 mM of SNAP incubation was selected for use in the following experiments. To further confirm the protective role of NO, LDH release and apoptotic nuclei were investigated. As shown in Figure 1B, C and D, compared with EFS alone-treated cells, SNAP treatment (1 mM) significantly reduced the increase in the content of LDH, the percentage of apoptotic nuclei, and the level of cleaved caspase-3 in EFS-stimulated cardiomyocytes.

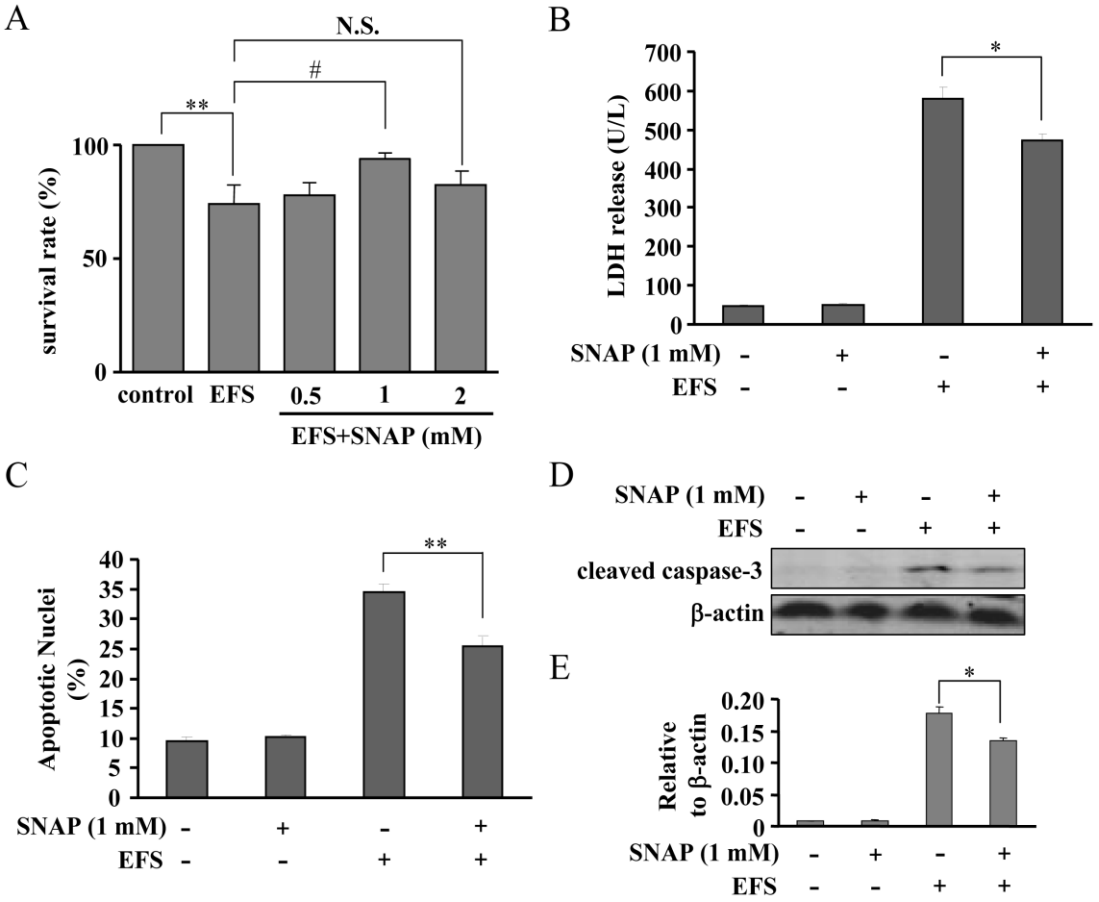

Figure 1. SNAP inhibits the apoptosis of primary cardiomyocytes induced by EFS. A. Statistical data showing that SNAP increased the survival rate of EFS-stimulated primary cardiomyocytes (n = 8, ***P* < 0.01, EFS *vs* control; ##*P* < 0.05, SNAP 1 mM *vs* EFS). N.S.: no significance. B, C. Statistical data showing that pretreatment of cardiomyocytes with SNAP (1 mM, 30 min) significantly reduced the increase in LDH content (B) and apoptotic nuclei (C) in EFS-stimulated cardiomyocytes (n = 8, **P* < 0.05, ***P* < 0.01, *vs* EFS alone). D. Representative images showing the effect of SNAP (1 mM) on the level of cleaved caspase-3 in EFS-stimulated cardiomyocytes. E. Statistical data showing the effect of SNAP on the level of cleaved caspase-3 in EFS-stimulated cardiomyocytes (n = 3, **P* < 0.05, *vs* EFS alone).

Effects of NO on the expression of Bax and Bcl-2 in EFS-stimulated cardiomyocytes

Because Bax and Bcl-2 can control the process of apoptosis, we measured the expression of Bax and Bcl-2 in EFS- and/or SNAP-treated cardiomyocytes. We found that the expression of Bax protein was significantly up-regulated in EFS-stimulated primary cardiomyocytes, but the expression of Bcl-2 protein was not, suggesting that EFS triggers apoptosis mainly by interrupting the expression of Bax (Figure 2A). Interestingly, SNAP (1 mM) treatment markedly reduced the increase in the expression of Bax protein in EFS-stimulated cardiomyocytes, but the expression of Bcl-2 protein was not changed (Figure 2A). These data were showed as the change in Bax/Bcl-2 protein ratio in EFS- and/or SNAP-treated cardiomyocytes (Figure 2B). The change of protein could be due to the alteration of either gene transcription or protein translation. To distinguish these possibilities, we monitored the formation of Bax and Bcl-2 mRNA in EFS-stimulated cardiomyocytes in the absence or presence of SNAP. As shown in Figure 2C, SNAP treatment (1 mM) decreased the formation of Bax mRNA in EFS-stimulated cardiomyocytes, while the formation of Bcl-2 mRNA was not changed. These data were also presented as the change in Bax/Bcl-2 mRNA ratio in EFS- and/or SNAP-treated cardiomyocytes (Figure 2D).

NO inhibits the apoptosis of primary cardiomyocytes induced by I/R

Because NO is protective in the process of cardiac ischemia/reperfusion (I/R), we further evaluated the role

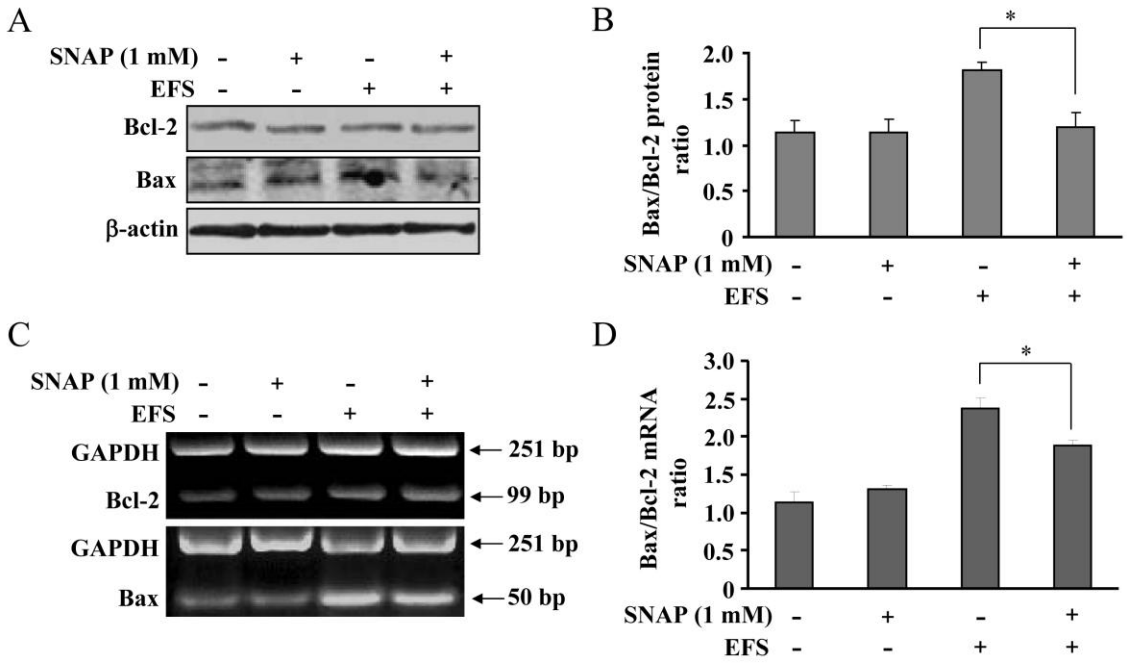

Figure 2. Effects of NO on the expression of Bax and Bcl-2 in EFS-stimulated cardiomyocytes. A. Representative images showing the effect of SNAP (1 mM, 30 min) on the change in the expression of Bax and Bcl-2 protein in EFS-stimulated cardiomyocytes. B. Statistical data showing the effect of SNAP (1 mM, 30 min) on Bax/Bcl-2 protein ratio in EFS-stimulated cardiomyocytes (n = 3, *P < 0.05, *vs* EFS alone). C. Representative images showing the effect of SNAP (1 mM, 30 min) on the change in the expression of Bax and Bcl-2 mRNA in EFS-stimulated cardiomyocytes. D. Statistical data showing the effect of SNAP (1 mM, 30 min) on Bax/Bcl-2 mRNA ratio in EFS-stimulated cardiomyocytes (n = 3, *P < 0.05, *vs* EFS alone).

of NO in I/R-induced cell injury. In this study, we found that I/R stimulation significantly decreased the survival rate of primary cardiomyocytes (Figure 3A). Pretreatment of cardiomyocytes with SNAP (1 mM, 30 min) partially reversed the decrease in the survival rate of I/R-stimulated cardiomyocytes, and this reversion was statistically significant at the concentration of 1 mM but not at 2 mM (Figure 3A). Compared with I/R alone-treated cells, SNAP treatment (1 mM) reduced the increase in the content of LDH, the percentage of apoptotic nuclei, and the level of cleaved caspase-3 in I/R-stimulated cardiomyocytes (Figure 3B to D).

Effects of NO on the expression of Bax and Bcl-2 in I/R-stimulated cardiomyocytes

We next measured the expression of Bax and Bcl-2 protein and mRNA in I/R- and/or SNAP-treated cardiomyocytes. We found that the expression of Bax protein and mRNA was significantly up-regulated in I/R-stimulated cardiomyocytes, but the expression of Bcl-2 was not, suggesting that I/R also induces apoptosis through interfering with the expression of Bax (Figure 4A, 4C). SNAP (1 mM) treatment reduced the increase in the expression of Bax protein and mRNA in I/R-stimulated cardiomyocytes, and the expression of Bcl-2 had no significant changes (Figure 4A, 4C). These data were showed as the change in Bax/Bcl-2 protein and mRNA ratio in EFS- and/or SNAP-treated cardiomyocytes (Figure 4B, 4D).

Effects of CaMKII activity in the protective role of NO in cardiomyocyte apoptosis

Because CaMKII was found to mediate the development of apoptosis, the role of CaMKII in the present study was detected. We first evaluated the involvement of CaMKII in EFS- or I/R-induced phosphorylation of PLB, a well-known substrate of CaMKII. As shown in Figure 5A to D, both EFS and I/R stimulation induced a marked increase in the phosphorylation level of PLB. Compared with EFS or I/R alone-treated cells, this increase was significantly reduced by SNAP incubation (1 mM, 30 min) (Figure 5A to D). Next, we evaluated the role of CaMKII activity in I/R-induced change in the expression of Bax and Bcl-2 protein using a specific inhibitor of CaMKII KN93. Our data showed that pretreatment of cardiomyocytes with KN93 (30 μM, 30 min) considerably reduced the increase

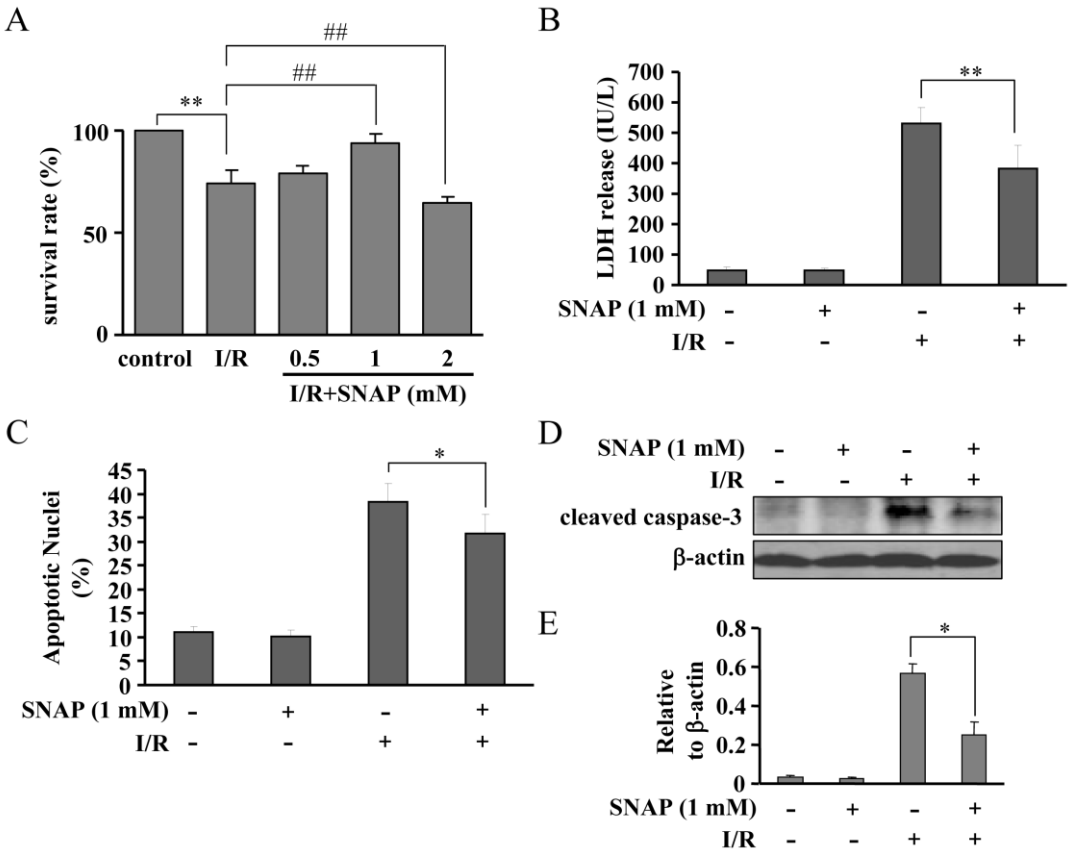

Figure 3. SNAP inhibits the apoptosis of primary cardiomyocytes induced by I/R. A. Statistical data showing that SNAP increased the survival rate of I/R-stimulated primary cardiomyocytes (n = 8, $^{**}P < 0.01$, I/R *vs* control; $^{\#\#}P < 0.05$, SNAP 1 mM or 2 mM *vs* I/R). B, C. Statistical data showing that pretreatment of cardiomyocytes with SNAP (1 mM, 30 min) significantly reduced the increase in LDH content (B) and apoptotic nuclei (C) in I/R-stimulated cardiomyocytes (n = 8, $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* I/R alone). D. Representative images showing the effect of SNAP (1 mM) on the level of cleaved caspase-3 in I/R-stimulated cardiomyocytes. E. Statistical data showing the effect of SNAP on the level of cleaved caspase-3 in I/R-stimulated cardiomyocytes (n = 3, $^{*}P < 0.05$, *vs* I/R alone).

in the expression of Bax protein in I/R-stimulated cardiomyocytes (Figure 5E). KN93 incubation did not alter the expression of Bcl-2 protein (Figure 5E). These data were also presented as the change in Bax/Bcl-2 protein ratio in I/R- and/or KN93-treated cardiomyocytes (Figure 5F).

DISCUSSION

It has been shown that NO can be released by the conversion of L-arginine to L-citrulline, a reaction catalyzed by one of three NO syntheses: neuronal NOS (Danson et al., 2005), endothelial NOS (Kolluru et al., 2010), and inducible NOS (Aktan, 2004). The interaction of NO with free superoxide radicals to form peroxynitrite ($ONOO^-$) arouses numerous concerns about the damaging effects of NO. However, in I/R, NO has also been shown to be beneficial because of its ability to maintain vascular permeability (Mayhan, 2000), prevent neutrophil infiltration (Shelton et al., 2008) and protect microvasculature integrity (López-Farré et al., 1998). In the present study, our data showed that SNAP inhibits the *in vitro* I/R-induced apoptosis of primary cardiomyocytes which was exemplified by the decrease in LDH release, the number of apoptotic nuclei and the level of cleaved caspase-3.

I/R injury is a common complex phenomenon encountered in clinical cases. Ischemia and reperfusion are actually inseparable. For instance, in aortic operation, the aorta needs to be clamped for hours before the clamp is removed and the blood returns. Paradoxically, reperfusion of ischemic tissue results in further injury but not protection (Boyle et al., 1997; Kaminski et al., 2002). This secondary injury is tightly associated with cell apoptosis. Hence, exploring the mechanism of apoptosis

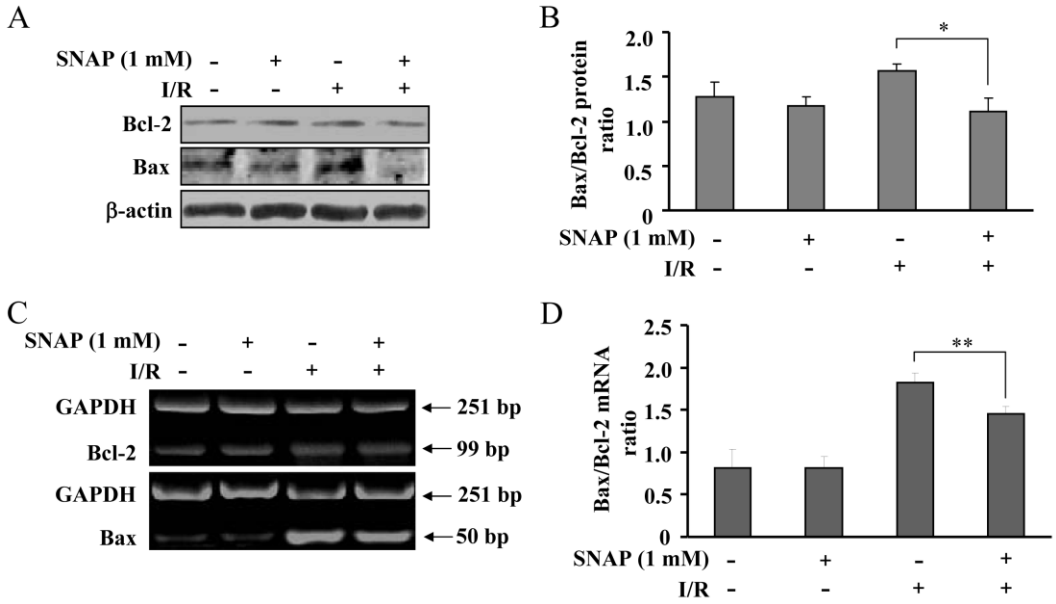

Figure 4. Effects of NO on the expression of Bax and Bcl-2 in I/R-stimulated cardiomyocytes. A. Representative images showing the effect of SNAP (1 mM, 30 min) on the change in the expression of Bax and Bcl-2 protein in I/R-stimulated cardiomyocytes. B. Statistical data showing the effect of SNAP (1 mM, 30 min) on Bax/Bcl-2 protein ratio in I/R-stimulated cardiomyocytes (n = 3, **P* < 0.05, *vs* I/R alone). C. Representative images showing the effect of SNAP (1 mM, 30 min) on the change in the expression of Bax and Bcl-2 mRNA in I/R-stimulated cardiomyocytes. D. Statistical data showing the effect of SNAP (1 mM, 30 min) on Bax/Bcl-2 mRNA ratio in I/R-stimulated cardiomyocytes (n = 3, ***P* < 0.01, *vs* I/R alone).

in cardiac I/R is pivotal to understand the heart ischemia. Apoptosis is a highly conserved and complex events characterized by cell shrinkage, chromatin condensation, nucleosomal DNA fragmentation and disruption of mitochondrial membrane potential resulting in plasma membrane disruption and apoptotic body formation (Allen et al., 1997). These morphological changes are believed to be caused in part by the imbalance of Bax and Bcl-2, two cytoplasm proteins that play crucial roles in executing apoptosis (Wiren et al., 2006; Hossini et al., 2008). Here, we found that I/R stimulation up-regulated the expression of Bax protein and mRNA. This up-regulation can be partially reversed by SNAP. However, the expression of anti-apoptosis protein Bcl-2 was not changed in both I/R alone- and I/R-SNAP-co-treated cells. Accordingly, the Bax/Bcl-2 ratio was reduced after SNAP incubation. It is well known that elevated Bax/Bcl-2 ratio corresponds to the onset of apoptosis (Wiren et al., 2006; Hossini et al., 2008). Hence, the decrease of Bax/Bcl-2 ratio might contribute to the protective effects of SNAP on *in vitro* I/R-induced cardiomyocyte apoptosis.

To further evaluate the protective role of NO in cardiomyocyte apoptosis, we used another stimulus EFS to stimulate cultured cardiomyocytes. EFS is an electrical stimulus that can be used to study the functions of different cells such as endothelium (Bai et al., 2004) and neuron (Ren et al., 2012). Although nanosecond pulsed electric fields (nsPEFs) is emerging as a novel stimulus for inducing apoptosis in various cancer cell lines *in vitro* (Ren et al., 2012; Stacey et al., 2003), here we first reported that non-nanosecond electric stimulation EFS induces cardiomyocyte apoptosis, which was confrimed by the increase in LDH release, apoptosis nuclei and cleaved caspase-3. In addition, EFS-induced apoptosis was also correlated with the increase in the expression of Bax protein and mRNA, and SNAP treatment reduced the EFS-induced increase in LDH release, apoptosis nuclei, and cleaved caspase-3 in cardiomyocytes. In conclusion, these data clearly indicated that EFS-induced apoptosis was similar to that induced by I/R. NO might protect cardiomyocytes from EFS-induced apoptosis.

To date, the mechanism for the EFS or I/R-induced cardiomyocyte apoptosis is still obscure. CaMKII is an important kinase that is involved in various processes. In mammalian cells, four CaMKII genes (α, β, γ, δ) give rise to a number of isoforms (Sandström et al., 2006). These isoforms are generally expressed and can be divided into mono- or multifunctional subunits. Functionally, CaMKII is a major mediator of cellular Ca^{2+} effects, and its biological actions have been studied in numerous cases such as learning and memory (Lucchesi et al., 2011) and ischemia (Vest et al., 2009). In apoptosis, CaMKII also exerts important functions. For example, previous studies reported that CaMKII inhibition reduces LDH release and

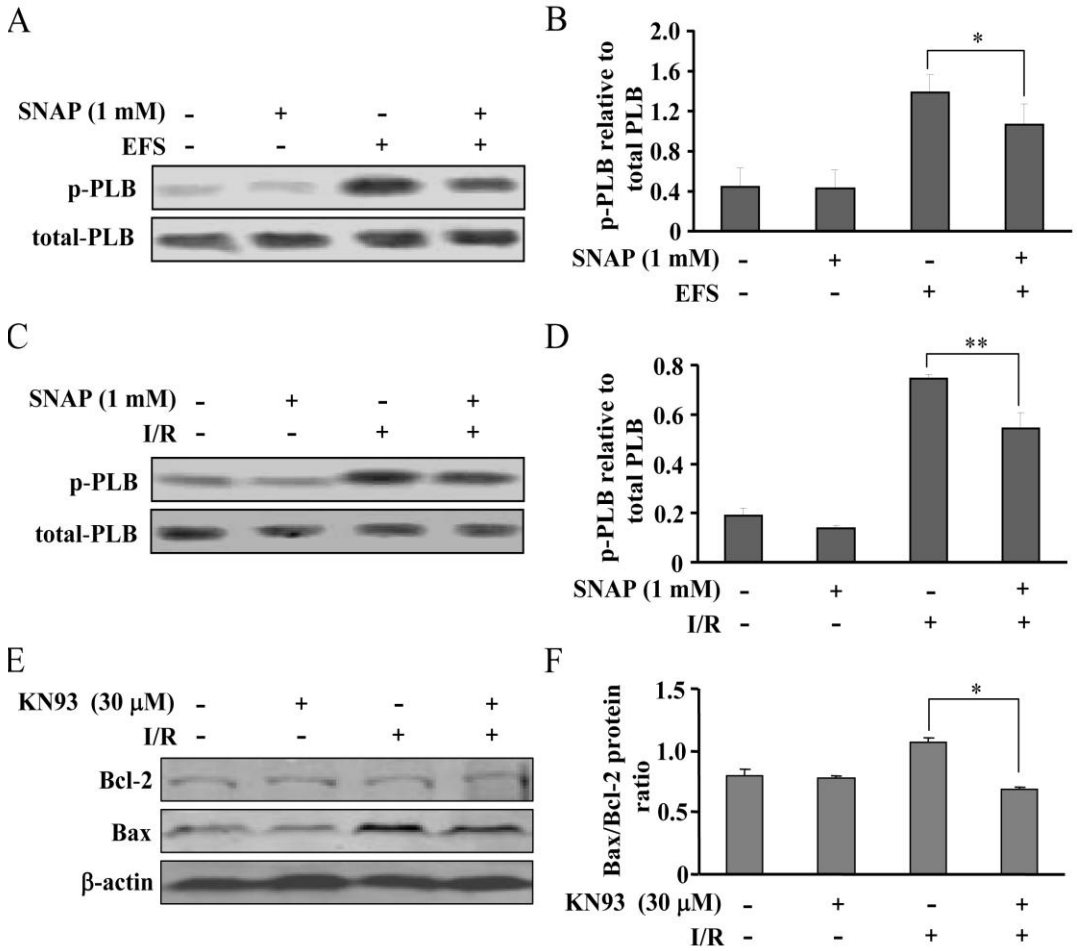

Figure 5. Effects of CaMKII activity in the protective role of NO in cardiomyocyte apoptosis. A. Representative images showing the effect of SNAP (1 mM, 30 min) on the change in the phosphorylation level of PLB in EFS-stimultated cardiomyocytes. B. Statistical data showing the effect of SNAP (1 mM, 30 min) on the change in the phosphorylation level of PLB in EFS-stimultaed cardiomyocytes (n = 3, **P* < 0.05, *vs* EFS alone). C. Representative images showing the effect of SNAP (1 mM, 30 min) on the change in the phosphorylation level of PLB in I/R-stimultaed cardiomyocytes. B. Statistical data showing the effect of SNAP (1 mM, 30 min) on the change in the phosphorylation level of PLB in I/R-stimultaed cardiomyocytes (n = 3, ***P* < 0.01, *vs* I/R alone). E. Representative images showing the effect of KN93 (30 μM, 30 min) on the change in the expression of Bax and Bcl-2 protein in I/R-stimultaed cardiomyocytes. F. Statistical data showing the effect of KN93 (30 μM, 30 min) on the change in Bax/Bcl-2 protein ratio in I/R-stimultaed cardiomyocytes (n = 3, **P* < 0.05, *vs* I/R alone).

Bax/Bcl-2 ratio (Vila-Petroff et al., 2006) and enforces expression of active CaMKII leading to apoptosis (Zhu et al., 2007).

These data cue us that CaMKII might be involved in the attenuation of NO in EFS- or I/R-induced cardiomyocyte apoptosis by reducing Bax expression. In fact, we found that EFS and I/R were found to elevate the phosphorylation level of PLB, indirectly showing the involvement of CaMKII activity in cardiomyocyte apoptosis. SNAP treatment reduced PLB phosphorylation in both EFS- and I/R-stimulated cardiomyocytes. In addition, we found that direct inhibition of CaMKII by KN93 exhibits similar effect on I/R-induced change in Bax and Bcl-2 expression. These data demonstrated that NO exerts protective functions in part by reducing the activity of CaMKII. This conclusion is supported by the fact that CaMKII activity can be inhibited through S-nitrosylation of CaMKII at cysteine 6 (Takata et al., 2011; Song et al., 2008).

Conclusion

NO reduces EFS- or I/R-induced apoptosis of primary cardiomyocytes likely by attenuating the activity of CaMKII.

This result provides us a potential opportunity to further understand the function of NO in cardiomyocyte apoptosis.

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Full Length Research Paper

Evaluation of saliva levels and therapeutic effects of morinidazole, a new 5-nitroimidazole derivative, in patients with periodontal diseases

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The aim of this study was to evaluate and compare the saliva and gingival crevice fluid concentrations, as well as therapeutic effects of morinidazole (MNZ) and ornidazole (ONZ) in patients with periodontitis after intravenous (i.v.) infusion administration. 142 outpatient subjects (80 women and 62 men) were enrolled in the phase II clinical trial, and the patients were randomly assigned to receive intravenous infusions of either Ornidazole or Morinidazole, twice daily for 5 days (500 mg each time, infusion period was 50 min). A saliva was collected prior to and again after finishing the first i.v. dose at intervals of 15, 30, 45, 60, 90 min on day 1. Bacteriological studies were undertaken to determine the effectiveness of the MNZ or ONZ. Reverse phase high performance liquid chromatography (RP-HPLC) method with UV detection at 318 nm was carried out, using a mixture of KH_2PO_4, acetonitrile and methanol (55:15:30, v/v/v) as a mobile phase, with a flow rate of 1.0 ml/min. There was no significant difference between the MNZ and ONZ groups. The cure rate of morinidazole was found to be slightly higher than that of ONZ, just like the result of concentration in saliva, but no significant differences between the two agents were apparent. During our trial, we found that the clinical resistance of anaerobes to morinidazole was proven to be extremely rare.

Key words: Periodontitis, morinidazole, ornidazole, tinidazole, saliva.

INTRODUCTION

Periodontal disease are generally considered to cause major oral health problems around the world. Specific anaerobic bacteria within the periodontal pocket are thought to be responsible for periodontal disease and as infection takes hold, a cascade of tissue- destructive pathways ensues, fuelled by inflammatory mediators (Ha et al., 2012)

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et al., 2012). Over 400 species of bacteria have been isolated from plaques (biofilms formed by bacteria), but only a few species of bacteria release toxins and induce a host inflammatory response resulting in the destruction of alveolar bone and connective tissues that support the dentition.

There is increasing evidence of an association between poor oral health and a number of clinically important medical conditions, such as cardiovascular disease, type 2 diabetes, respiratory disease, kidney disease and adverse pregnancy outcomes (Newnham et al., 2009; Shub et al., 2009; Ford et al., 2010). The therapeutic goal is to remove bacteria that is responsible for the infection by both mechanical cleaning and the application of antimicrobial agents, such as tetracycline, 5-nitroimidazole derivatives, clindamycin, ofloxacin, etc (Perioli et al., 2004). Tetracyclines, in particular doxycycline, are used extensively in the treatment of periodontal disease, but the development of bacterial resistance has led to a preference for the use of 5-nitroimidazole derivatives which are very selective agents against anaerobic bacteria (Patel et al., 2008).

Metronidazole (MEZ) is the first generation member of 5-nitroimidazole derivative with bactericidal activity against most anaerobic bacteria and protozoa. It is now used as the principal treatment for *Helicobacter pylori* infections, amebiasis, giardiasis, trichomoniasis, bacterial vaginosis, Crohn's disease and as a prophylactic antibiotic in surgical interventions. It is also a widely used antibacterial compound in the treatment of some types of periodontal diseases. MEZ, one of the most globally used drugs, has been used for over 43 years. It is among the top 100 most frequently prescribed drugs in the US and among the top 10 during pregnancy (Akay et al., 2003).

Tinidazole (TNZ) and Ornidazole (ONZ) are both second and third generation members of the 5-nitroimidazole groups with selective activity against anaerobic bacteria and protozoa, such as *Tichomonas vaginalis*, *Entamoeba histolytica* and *Gardia lamblia*. They can also be used effectively against metronidazole-resistant strains of *T. vaginalis* and recurrent periodontitis (Mombelli et al., 2005; Zhang et al., 2006). Tinidazole has been widely used in Europe and developing countries for over two decades with established efficacy and acceptable tolerability (Fung and Doan, 2005). Tinidazole is also effective in the treatment of respiratory tract infections, intra-abdominal sepsis, obstetrical and gynecological infections, colonic and abdominal surgery, emergency appendectomy and amebic liver abscess, etc (Austin et al., 2006; Bakshi and Singh, 2004).

With the development of bacterial resistance, there is an increasing need for R&D of new 5-nitroimidazole derivatives as very selective agents against anaerobic bacteria. Morinidazole (MNZ) (Figure 1) is a new synthetic bacterial infections, such as periodontal diseases and gynecological diseases. It is currently in Phase III Clinical Trials (Lv, 2010). The aim of the present study is to evaluate and compare the saliva concentrations and therapeutic effects of MNZ to ONZ in patients with periodontitis after i.v. infusion administration.

Ethical approval

Our study was approved by the Ethics Committee of the first affiliated Hospital with Nanjing Medical University, Nanjing, China.

MATERIALS AND METHODS

Study population and study groups

The study population consisted of 142 outpatient subjects (80 women and 62 men) attending the Department of Periodontics in the first affiliated hospital with Nanjing Medical University and the first affiliated hospital with Shanghai Jiao Tong University, China. Written consent was obtained from those who agreed to participate voluntarily and ethical clearance was obtained from the Hospital's ethical committee (Table 1). Periodontal disease was defined as the presence of periodontal pockets ≥ 4 mm in depth at 12 or more probing sites in fully erupted teeth (typically excluding wisdom teeth) (Hart et al., 2012). Patients with a habit of smoking, gross oral pathology, heart diseases, respiratory diseases, tumors or any other systemic disease, which can alter the course of periodontal disease, were excluded. Furthermore patients on any medication like phenytoin, cyclosporins, anti-inflammatory, antibiotics, or who had received periodontal therapy in the preceding 2 weeks, as well as pregnant and lactating females, were excluded from the study. Each subject underwent a full mouth periodontal probing and charting, along with the periapical radiographs.

Experimental protocol

The study was conducted in a double-blind fashion in which patients were randomly assigned to one of the two antimicrobial treatment groups. Certain rules were defined at the outset, and these were followed throughout the study. No patient would receive other systemic or local antimicrobial treatments about individual teeth. After the completion of the necessary scaling and root planning, the patients would receive intravenous infusions of either Ornidazole or Morinidazole, twice daily for 5 days. The dosage was both 500 mg each time, and the infusion period was 50 min. The packaging and labeling of the various medications were performed by persons who were not involved in the treatment or clinical decision-making. These medications were provided in coded fashion to the clinical team so as to maintain the double-blind nature of the study.

Collection of saliva

Saliva (1.0 ml) was collected from the gingival crevice fluid by aspiration through a capillary micropipette into 1.5 ml tubes prior to and again after finishing the first i.v. dose of Ornidazole or Morinidazole at intervals of 15, 30, 45, 60, 90 min on day 1. After collection, the saliva samples were placed immediately on ice and aliquoted before freezing at -70°C until analyzed.

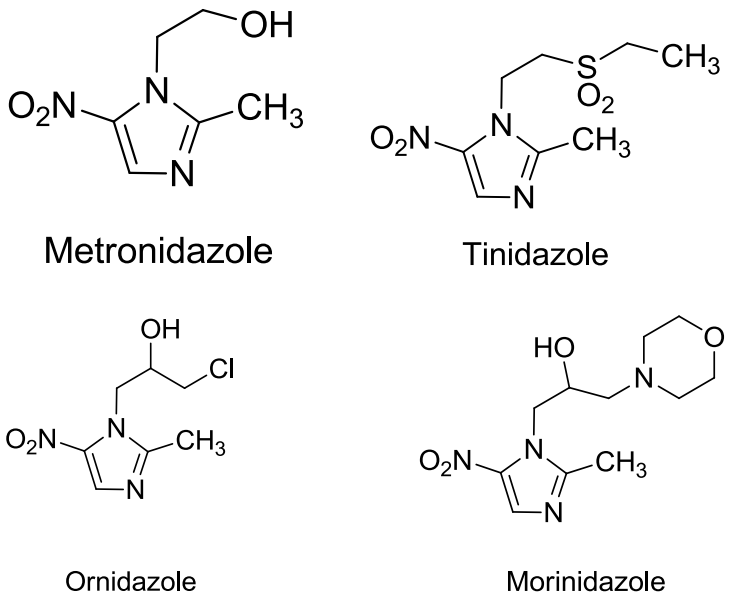

Figure 1. Chemical structure of metronidazole (MEZ, a), tinidazole (TNZ, b), ornidazole (ONZ, c) and morinidazole (MNZ, d).

Table 1. Demographic characteristic of treatment groups.

Characteristic		Morinidazole (n=71)	Ornidazole (n=71)
Age (years)		36.1±12.2 (20-61)	34.9±11.6 (23-65)
Gender	Men	30	32
	Women	41	39
Height (cm)		166.9±7.4 (155-183)	165.8±8.1 (154-181)
Body Weight (kg)		59.0±10.0 (46-85)	58.6±10.5 (47-81)
Body mass index (BMI)		21.1±2.7 (17.3-27.5)	22.1±2.5 (18.0-28.7)

Determination of ornidazole or morinidazole in saliva samples

Morinidazole or ornidazole saliva levels were measured by high-performance liquid chromatography (HPLC) with UV detection. Morinidazole (Purity 99.6%, Lot 20050805) was supplied by Hansoh Pharmaceutical Group, China. TNZ was used as the internal marker for each sample (ONZ and TNZ were supplied by the National Institute for the Control of Pharmaceutical and Biological Products, China). The assay was performed as follows: A 100 µl aliquot of human saliva sample was transferred to a 1.5 ml test tube into which 400 µl methanol was spiked. After vortex for 2 min and then centrifuge at 15,000 rpm for 10 min, the supernatant was transferred to an autosampler vial and 20 µl was injected into the HPLC system (LC-2010-CTH; Shimadzu, Kyoto, Japan) equipped with a Kromasil 100-5 C18 column (250 × 4.6 mm, 5 µm, Sweden). The mobile phase consisted of potassium dihydrogen phosphate (0.02 mol/l), acetonitrile and methanol (55:15:30, v/v/v, pH4.51). The flow rate was 1.0 ml/min, and the detector wave was set at 318 nm. Retention time was approximately 4.0, 4.5 and 5.6 min for TNZ, MNZ and ONZ, respectively. The peaks of interest were well separated and free from interference of endogenous substances. Calibration curves were plotted over the concentration range of MNZ (25.4 to 5080.0 ng/ml) and ONZ (25.0 to 5000.0 ng/ml). Both of the intra- and inter-batch relative standard deviations (RSD) were less than 10.0%.

Bacteriological studies

Bacteriological studies were undertaken to determine the effectiveness of MNZ or ONZ *in vitro*, which was performed as follows: *Peptostreptococcus anaerobius*, *Veillonella parvula*, *Actinomyces dentocariosus*, *Porphyromonas gingivalis*, *Bacteroides thetaiotaomicron* and *Prevotella melaninogenica* were maintained under anaerobic conditions at 37°C for 48 h. Five to 10 well-isolated colonies were removed from brain heart infusion (BHI) plates, transferred to 5 ml of BHI broth, vortex, and incubated at 37°C for 48 h under anaerobic conditions (80% nitrogen, 10% hydrogen, and 10% carbon dioxide). Then, the cell suspension was standardized to match the turbidity of the 1.0 McFarland Standards by using a

Table 2. Minimum inhibitory concentration (MIC) of MNZ and ONZ to several anaerobic bacteria.

Species of anaerobic bacterium	Morinidazole (mg/L)	Onidazole (mg/L)
Peptostreptococcus anaerobius (n=2)	0.25-1.0	0.25-1.0
Veillonella parvula (n=4)	0.31±0.13 (0.25-0.5)	0.31±0.13(0.25-0.5)
Actinomyces dentocariosus (n=1)	0.25	0.25
Porphyromonas gingivalis (n=1)	2.00	2.00
Bacteroides thetaiotaomicron (n=1)	4.00	4.00
Prevotella melaninogenica (n=1)	0.5	0.5

Table 3. The concentration of MNZ and ONZ in saliva after finishing a 50-min period i.v. infusion administration at the following time point of 15, 30, 45 and 60 min.

Time (min)	Concentration (ng/ml)	
	MNZ (n=34)	ONZ (n=29)
15	4267.5±2307.2	4108.1±2388.1
30	4199.1±2224.9	3759.8±2492.9
45	3972.3±2155.1	3654.4±2203.7
60	3842.0±2421.3	3428.7±2398.7

microscan turbidity meter (LeCorn et al., 2007). The resulting suspensions contained approximately 3×10^8 colony forming units/ml per sample. Four-millimeter thick Brucella agar plates supplemented with 5% defibrinated sheep blood, 1% vitamin K, and 0.5% hemin were inoculated by streaking a cotton swab, previously submerged in the bacterial suspension, over the entire agar surface. This procedure was repeated twice, rotating the plate 60° each time to ensure even distribution of the inoculum. Lastly, the rim of the agar was swabbed. After 10 min, antimicrobial test strips were positioned in the center of the plates. The plates were inverted and incubated anaerobically at 37°C for 48 to 72 h. The MIC was measured by two independent investigators.

Statistical analysis

All the data was analyzed using a software program (SPSS version 10.5, SPSS Inc., USA). The differences before and after each treatment regimen were also compared across the two treatment groups by one-factor analysis of variance (ANOVA). Paired t-test was used to compare MNZ or ONZ concentrations in saliva, which pair differ significantly at 5% level of significance.

RESULTS

Clinical trial results

After administration of the drugs, the main index of the clinical cure rate in full analysis set (FAS) of MNZ and ONZ groups was 92.9 and 91.5%, respectively. Total anaerobic bacteria clearance of MNZ and ONZ was 100.0 and 91.3%, respectively. The full recovery rate of MNZ and ONZ was just the same as that of total anaerobic bacteria clearance. The minimum inhibitory concentration (MIC) of MNZ and ONZ to several anaerobic bacteria is shown in Table 2. There is no significant difference between MNZ and ONZ in clinical cure rate, as well as total anaerobic bacteria clearance and also the full recovery rate. After administration of the drugs, the main index of the clinical cure rate in per protocol set (PPS) of MNZ and ONZ groups were both 100.0%. Total anaerobic bacteria clearance of MNZ and ONZ were 100.0 and 91.3%, respectively. The full recovery rate of MNZ and ONZ were both 100%. The result of PPS was kept in accordance with that of FAS.

Concentration of MNZ and ONZ in saliva

After finishing a 50 min period i.v. infusion of MNZ and ONZ for 15 min, both of them reached almost the same saliva level at more than 4100 ng/ml, then the concentration dropped moderately. The mean saliva concentration of MNZ was a litter higher than that of ONZ, but there was no significant statistical difference between them (Table 3).

DISCUSSION

Periodontal diseases are groups of infections and inflammatory conditions, including gingivitis and periodontitis that affect teeth-supporting structures. These diseases occur when bacteria from dental plaque invade

the surrounding tissues and from the accumulation of plaque at the gingival margin which induces an inflammatory response (Hart et al., 2012). Periodontal diseases are one of the many potential risk factors for systemic conditions and can affect their onset and progression by various mechanisms (Ebersole, 2003; Katancik et al., 2005). It has proved that there is a moderate association between cardiovascular diseases and periodontal diseases (Beck and Offenbacher, 2005). The risk factors for systemic diseases and the initiation or progression of these medical conditions can be reduced by the use of general medicine. The use of antimicrobials in the treatment of periodontal infections should be based on clinical symptoms and subsequent bacteriologic diagnosis. The systemic antimicrobial agents are used only when the clinical condition indicates that access surgery is a likely treatment procedure (Pähkla et al., 2005). Morinidazole has a broad spectrum for anaerobic bacteria and penetrates well into gingival crevice fluid, which makes it the drug of choice for anaerobic periodontal infections. The present findings confirm that morinidazole shows a satisfactory cure rate after 5 days of treatment. The concentration of MNZ in crevice fluid is about 38.7% to the peak plasma concentrations after been given by same i.v dosage infusion for 50 min, reaching a saliva level of more than 4100 ng/ml, which can inhibit or kill the *Bacteroides thetaiotaomicron* (the MIC of *B. thetaiotaomicron* was 4000 ng/ml). We find that many anaerobic bacteria are sensitive to the MNZ or ONZ, such as *Peptostreptococcus anaerobius*, *Veillonella parvula* and *Actinomyces dentocariosus*. All of them have the same MIC of 250 ng/ml, which is far below the peak saliva concentration of MNZ and ONZ. Clinical resistance of anaerobes to morinidazole is extremely rare.

In this study, the concentration of MNZ and ONZ in saliva reached peak levels and steady for about 45 min after finishing a 50 min period i.v. infusion and the variability of the concentrations is very large. The big variability of MNZ or ONZ concentration in saliva within the patients can be attributed to the different secretion status of individual patients at the time of collection of saliva sample. The cure rate of morinidazole is slightly higher than that of ONZ, as the result of concentration in saliva, but no significant differences between the two agents are apparent.

An antimicrobial dose of MNZ at 500 mg has been found to be both safe and effective when given twice daily over 5 days in randomized controlled clinical trials. The incidence rate of adverse events occurring in MNZ or ONZ groups is 5.6 and 2.8%, respectively. All the adverse events are mild, and all subjects complete the trial. The main adverse effects relate to the drugs are dizziness, drowsiness and nausea, etc.

The system used to deliver the drug should maintain the concentration of antibiotic for sufficient duration to reduce the anaerobic population in the subgingival ecosystem. Reduction in the Gram-negative anaerobic populations is frequently accompanied by an increase in aerobic or microaerophilic species such as *Streptococci* and also *Actinomyces* species. It is important that we should pay more attention to this phenomenon during the next clinical trials and clinical application. By greater integration of medicine and dentistry, dentists can take more responsibility for the management of their patients' systemic health and conversely the physicians can assume a more active role in their patients' oral health (Pradeep et al., 2009).

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Conflict of interest

The authors have no conflicts of interest to disclose.

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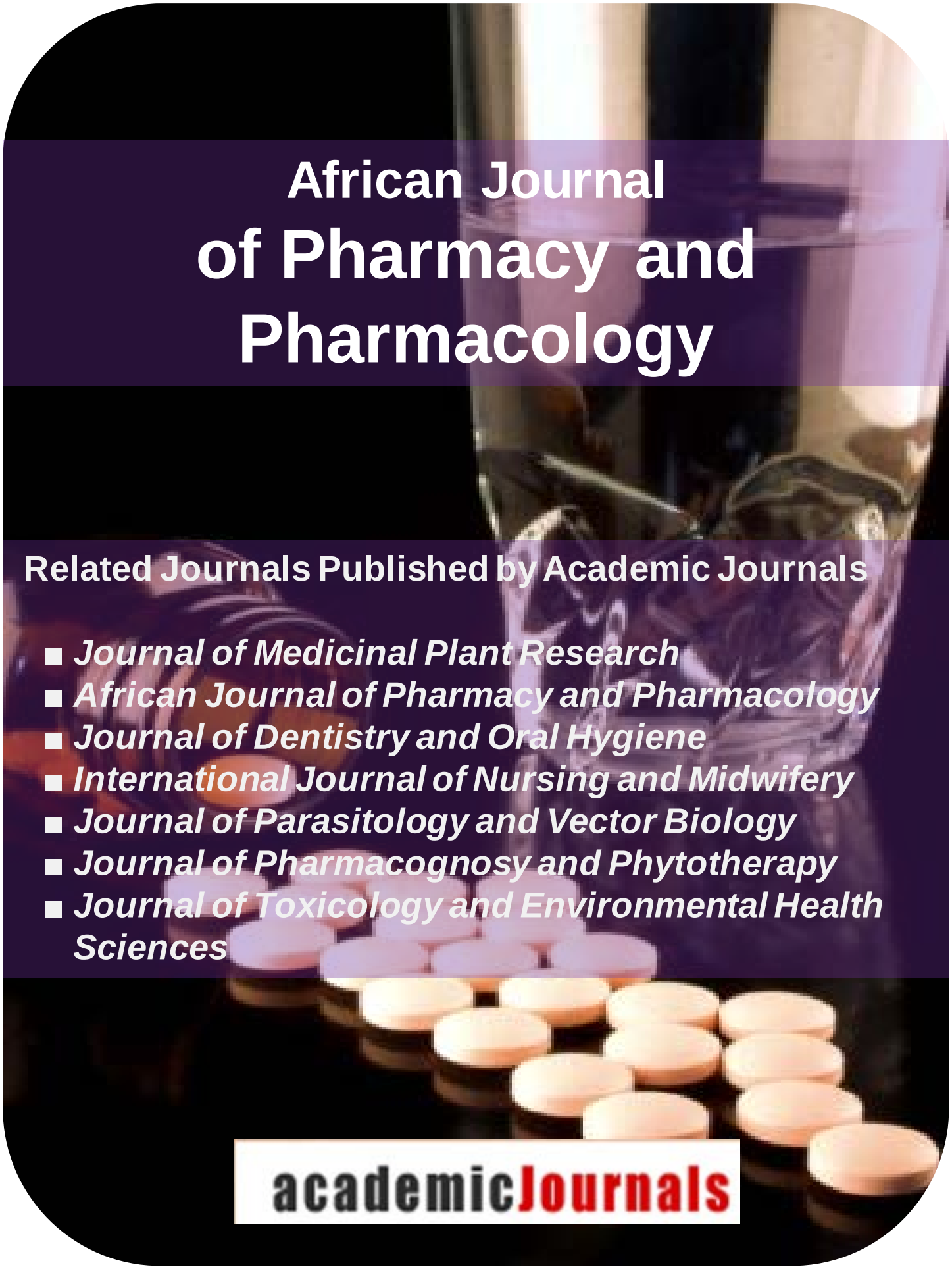

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