

Iodine-131 induces apoptosis in human cardiac muscle cells through the p53/Bax/caspase-3 and PIDD/caspase-2/t-BID/cytochrome *c*/caspase-3 signaling pathway

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Abstract. The aim of this study was to elucidate the effects of iodine-131 on the induction of apoptosis in human cardiac muscle cells and the underlying molecular mechanisms. We found that iodine-131 reduced cell proliferation, induced apoptosis, induced p53, PIDD, t-BID (mitochondria) protein expression, suppressed cytochrome *c* (mitochondria) protein expression, and increased Bax protein expression, and promoted caspase-2, -3 and -9 expression levels in human cardiac muscle cells. Meanwhile, si-p53 inhibited the effects of iodine-131 on the reduction in cell proliferation and induction of apoptosis in human cardiac muscle cells through regulation of Bax/cytochrome *c*/caspase-3 and PIDD/caspase-2/t-BID/cytochrome *c*/caspase-3 signaling pathway. After si-Bax reduced the effects of iodine-131, it reduced cell proliferation and induced apoptosis in human cardiac muscle cells through the cytochrome *c*/caspase-3 signaling pathway. However, si-caspase-2 also reduced the effects of iodine-131 on the reduction of cell proliferation and induction of apoptosis in human cardiac muscle cells through the t-BID/cytochrome *c*/caspase-3 signaling pathway. These findings demonstrated that iodine-131 induces apoptosis in human cardiac muscle cells through the p53/Bax/caspase-3 and PIDD/caspase-2/t-BID/cytochrome *c*/caspase-3 signaling pathway.

Introduction

In recent years, research regarding the role of cell apoptosis in cardiovascular diseases has advanced the understanding of numerous cardiovascular diseases (1). It was previously believed

that myocardial ischemia, as well as ischemia/reperfusion (IR) injury-induced myocardial death is a type of necrosis (2). It was demonstrated in recent research that oxidative stress, overloading, ischemia, hypoxia, and reperfusion injury will not only induce myocardial necrosis, but also cause myocardial apoptosis (3). Myocardial ischemia, ischemia/reperfusion, and heart failure are all related to myocardial apoptosis (3). As cell apoptosis is a pathological process controlled by a series of programs and mediated by signaling pathways, blocking its signaling pathways will contribute to blocking apoptosis, preventing a reduction in the number of myocardial cells, and sustaining or improving cardiac function (4).

Since the discovery that the caspase-3 protein, which promotes cell apoptosis in mammals and nematodes, possesses high homology in 1993, research has focused on the mechanism of action of such proteases on cell apoptosis. The caspase family plays a major role in apoptosis, among which, caspase-3 is the final effector of the caspase cascade that is involved in apoptosis, which plays a core role in the protease cascade cutting process. Various proteases will cut the caspase-3 zymogen, which activates caspase-3, and the activated caspase-3 will further cut various substrates, resulting in final cell apoptosis (5). It is the marker as well as the executor of cell apoptosis, the major mechanism of which is to digest and destroy multiple intracellular protease complexes, activate the intranuclear nuclease, lead to DNA pyrolysis, destroy the calcium pump function of cells, resulting in intracellular calcium overload (6).

p53 gene expression plays a leading role in the myocardial apoptosis of IR. Myocardial apoptosis in hypoxia-induced newborn rats is markedly increased, and expression of both p53 protein and mRNA is notably increased, as can be found in research on cultured myocardial cells in newborn rats (7). Bcl-2 gene expression can block the p53-induced cell apoptosis, which is independent of changes in p53 expression and its location in the nucleus; in addition, it can inhibit the bax transcription of p53 (7,8). Bcl-2 is an important anti-apoptotic factor (9).

Iodine-131 is a type of radionuclide dominated by γ - and β -decay, and is used to kill multi-layer tumor cells and cover the leukemia proliferation (10). During the process of applying

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iodine to treat differentiated thyroid carcinoma, accompanying with cell morphological and functional degeneration; besides, the expression of sodium-iodine symporter and thyroid-stimulating hormone receptor gene is reduced, with the decrease or even the loss of iodine uptake capacity (11). In the present study, we investigated the effects of iodine-131 on the induction of apoptosis in human cardiac muscle cells and the underlying molecular mechanisms.

Materials and methods

Cell culture and cell viability assay. H9c2 cells were provided by Shanghai Cell Bank (Shanghai, China) and maintained in complete Dulbecco's modified Eagle's medium (DMEM) at 37°C in a humidified incubator containing 5% CO₂. The cells were seeded in 96-well plates for 24 h with culture medium per well containing 7.4, 14.8 and 29.6 MBq/ml of iodine-131 for 24, 48 and 72 h. Following addition of 40 μ l/well MTT, the cells were incubated for 4 h and the medium was discarded. DMSO (150 μ l/well) was added and incubated for 20 min. The sample absorbance was measured using a Multiskan plate reader (BioTek Instruments, Inc., Winooski, VT, USA) at 570 nm.

Cell apoptosis assay. The cells were seeded in 96-well plates for 24 h with culture medium per well containing 7.4, 14.8 and 29.6 MBq/ml of iodine-131 for 48 h. Subsequently, the cells were washed with phosphate-buffered saline (PBS) and centrifuged at 200 x g for 5 min. The cells were stained with 100 μ l of Annexin V-PI labeling solution (BD Biosciences, San Jose, CA, USA) for 15 min in darkness. The apoptosis rate was analyzed by flow cytometry (BD Biosciences).

Caspase-2/-3/-9 activity colorimetric protease assay. Cells were harvested and lysed in RIPA lysis buffer, and then protein content was extracted using the radioimmunoprecipitation assay buffer (Wolsen, Xi'an, China). Equal amount of protein was incubated with caspase-2/-3/-9 activity colorimetric protease assay for 2 h at 37°C. The sample absorbance was measured using a Multiskan plate reader (BioTek Instruments, Inc.) at 405 nm.

Western blot analysis. Cells were harvested and lysed in RIPA lysis buffer, and then the protein content was extracted using radioimmunoprecipitation assay buffer (Wolsen). Equal amount of protein was electrophoresed using 8-10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by transfer of the proteins to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membranes were blocked with 5% bovine serum albumin in TBAT (MP Biomedicals LLC, Santa Ana, CA, USA) for 1 h at room temperature and incubated overnight at 4°C with Bax, cytochrome c, PIDD, t-BID, cytochrome c and GAPDH (Santa Cruz Biotechnology, Inc.). Subsequently, the membranes were washed with TBAT and incubated with horseradish peroxidase-labeled anti-rabbit or anti-mouse immunoglobulin (Ig)G (Santa Cruz Biotechnology, Heidelberg, Germany) at 37°C for 1 h.

Statistical analysis. Data are presented as the mean \pm standard deviation. Experimental results were assessed using

Chi-square test, t-test or ANOVA as appropriate. Results were considered significant at $p \leq 0.05$.

Results

Iodine-131 reduces cell proliferation and induces apoptosis in human cardiac muscle cells. To determine whether iodine-131 affects human cardiac muscle cell growth, MTT assay and flow cytometry were used to assess cell proliferation and apoptosis, respectively. As showed in Fig. 1A, 30 MBq/ml of iodine-131 significantly reduced cell proliferation and induced apoptosis of human cardiac muscle cells at 24, 48 and 72 h. Meanwhile, 20 MBq/ml of iodine-131 significantly reduced cell proliferation and induced apoptosis of human cardiac muscle cells at 48 and 72 h (Fig. 1A). Moreover, 10 MBq/ml of iodine-131 significantly reduced cell proliferation and induced apoptosis of human cardiac muscle cells at 72 h (Fig. 1). Iodine-131 (20 and 30 MBq/ml) significantly induced the apoptosis of human cardiac muscle cells (Fig. 1B).

Iodine-131 induces p53, PIDD, t-BID and Bax protein expression, and inhibits cytochrome c protein expression in human cardiac muscle cells. In order to investigate the mechanism involved in the human cardiac muscle cell apoptosis induced by iodine-131, we used western blot analysis to analyze p53, PIDD, t-BID and Bax protein expression, and cytochrome c protein expression in human cardiac muscle cells. As showed in Fig. 2, iodine-131 significantly induced p53, PIDD, t-BID and Bax protein expression, and decreased cytochrome c protein expression in human cardiac muscle cells.

Iodine-131 promotes caspase-2, -3 and -9 expression levels in human cardiac muscle cells. To determine the mechanism underlying the effects of iodine-131 on caspase-2, -3 and -9 expression levels in human cardiac muscle cells, commercial kits were used to analyze caspase-2, -3 and -9 expression levels. As showed in Fig. 3, iodine-131 significantly promoted caspase-2, -3 and -9 expression levels in human cardiac muscle cells.

si-p53 inhibits the effects of iodine-131 on cell proliferation and apoptosis in human cardiac muscle cells. To assess the function of p53 on the effects of iodine-131 on human cardiac muscle cell apoptosis, si-p53 was used to inhibit p53 expression in human cardiac muscle cells. Fig. 4 indicates that si-p53 significantly reversed the effects of iodine-131 and increased cell proliferation and inhibited apoptosis of human cardiac muscle cells following treatment with 20 MBq/ml of iodine-131.

si-p53 inhibits the effects of iodine-131 on the regulation of the PIDD/caspase-2/t-BID/caspase-3 signaling pathway in human cardiac muscle cells. In addition, we investigated whether p53 is involved in the effects of iodine-131 on apoptosis. Figs. 5A, C, E and F and 6 show that si-p53 significantly inhibited p53, PIDD, t-BID protein expression and caspase-2 expression levels in human cardiac muscle cells treated with 20 MBq/ml of iodine-131.

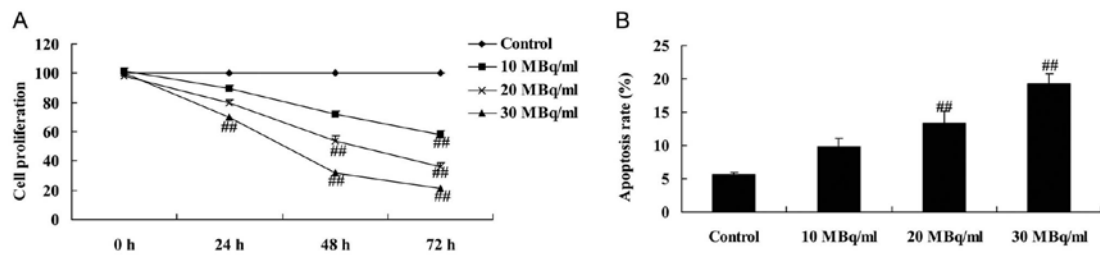


Figure 1. Iodine-131 reduces cell proliferation, and induces apoptosis in human cardiac muscle cells. Iodine-131 reduced cell proliferation (A), and induced apoptosis (B) in human cardiac muscle cells. $^{##}p < 0.01$ vs. the control group.

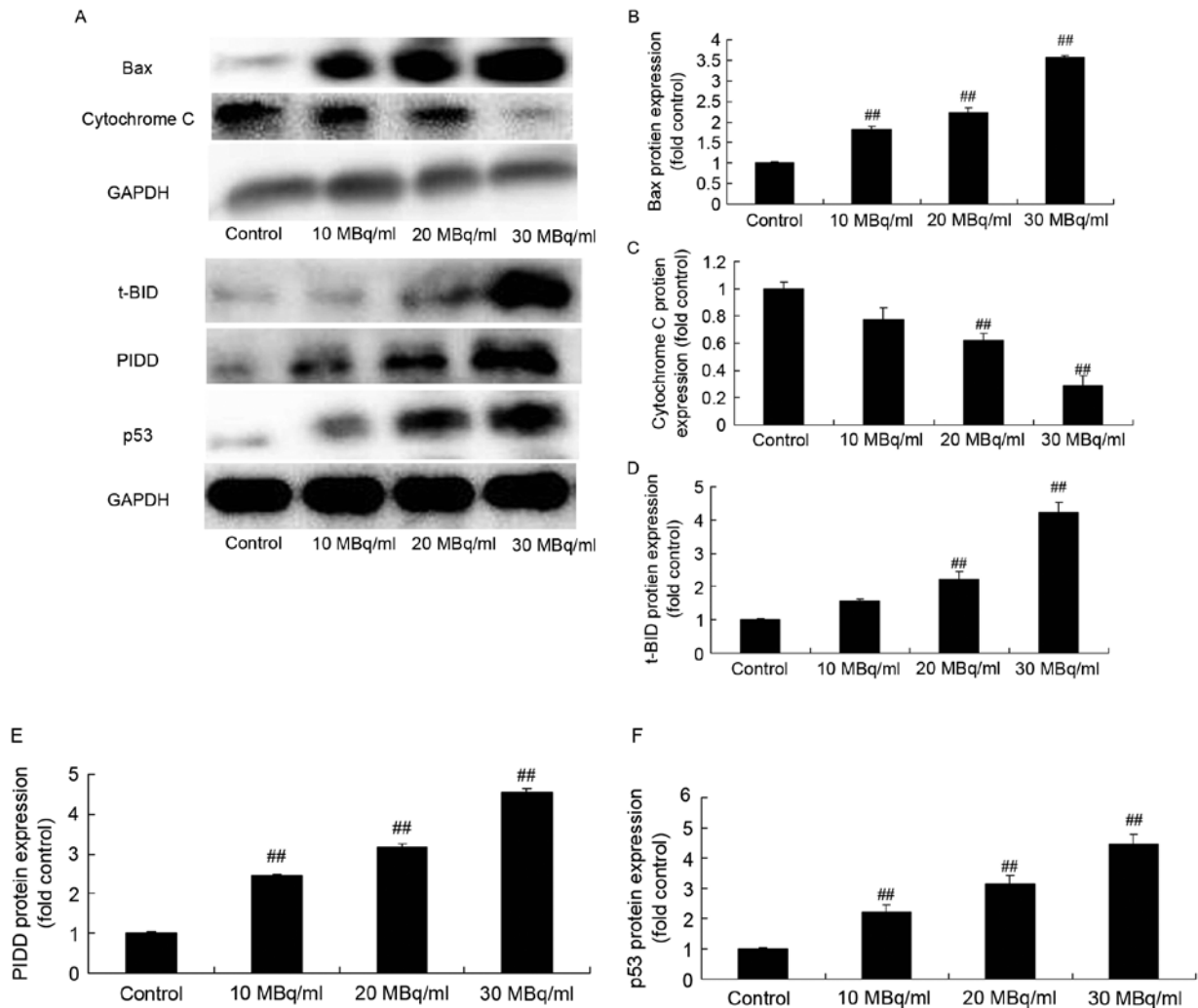


Figure 2. Iodine-131 induces p53, PIDD, t-BID and Bax protein expression, and inhibits cytochrome *c* protein expression in human cardiac muscle cells. p53, PIDD, t-BID, Bax and cytochrome *c* protein expression as determined using western blot analysis (A), and statistical analysis (B-F) in human cardiac muscle cells. $^{##}p < 0.01$ vs. the control group.

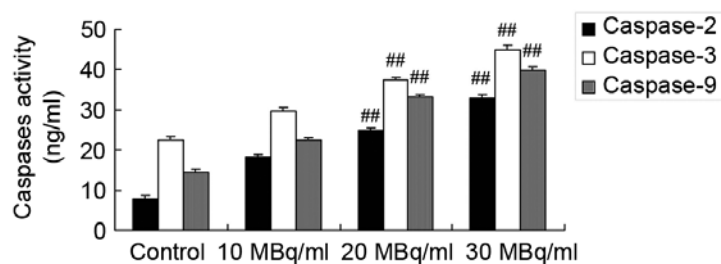


Figure 3. Iodine-131 promotes caspase-2, -3 and -9 expression levels in human cardiac muscle cells. $^{##}p < 0.01$ vs. the control group.

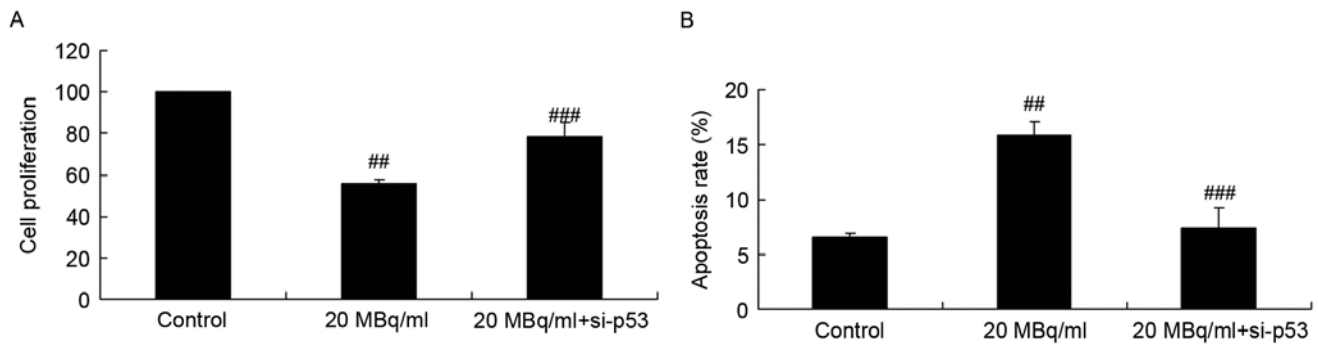


Figure 4. si-p53 inhibits the effects of iodine-131 on cell proliferation and apoptosis in human cardiac muscle cells. si-p53 inhibited the effects of iodine-131 on cell proliferation (A) and apoptosis (B) in human cardiac muscle cells. ## $p < 0.01$ vs. the control group, ### $p < 0.01$ vs. 20 MBq/ml iodine-131 group.

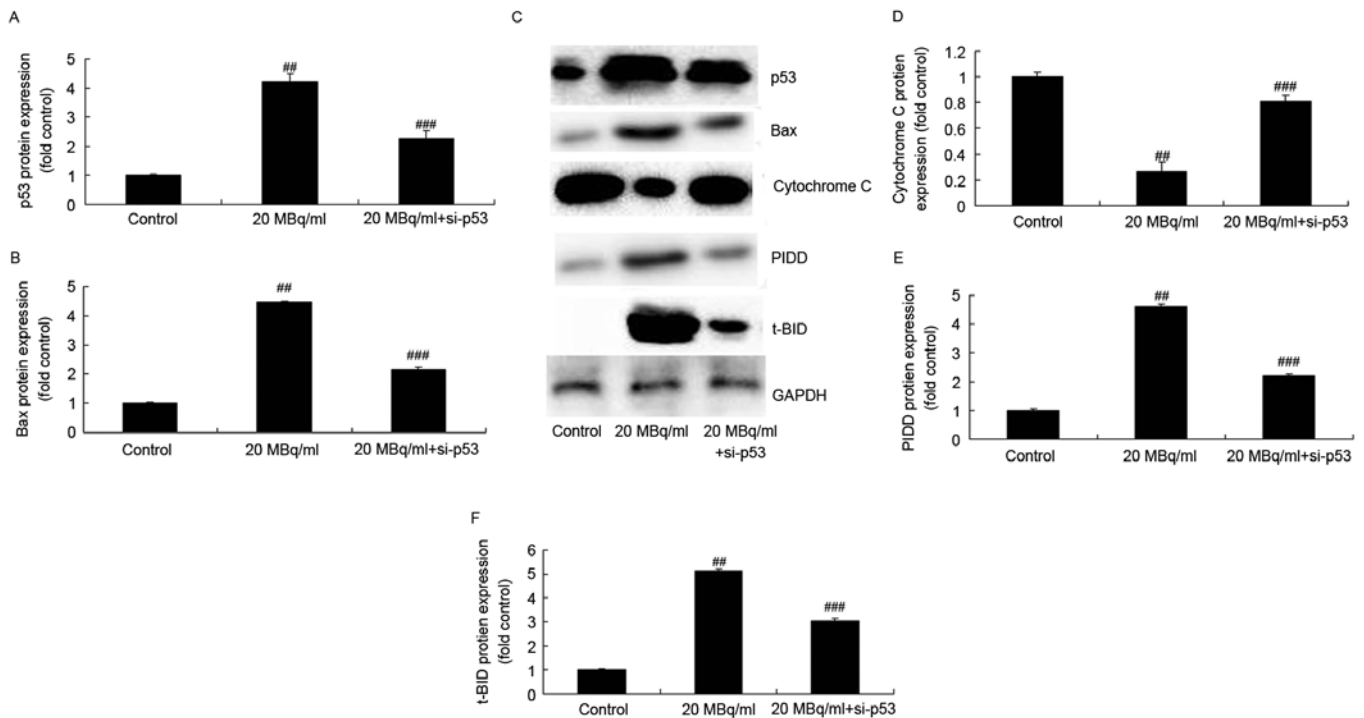


Figure 5. si-p53 inhibits the effects of iodine-131 on regulation of the PIDD/caspase-2/t-BID/cytochrome *c* signaling pathway in human cardiac muscle cells. p53, PIDD, t-BID, Bax and cytochrome *c* protein expression as detected using western blot analysis (C), and statistical analysis (A, B, D, E and F) in human cardiac muscle cells. ## $p < 0.01$ vs. the control group, ### $p < 0.01$ vs. 20 MBq/ml of iodine-131 group.

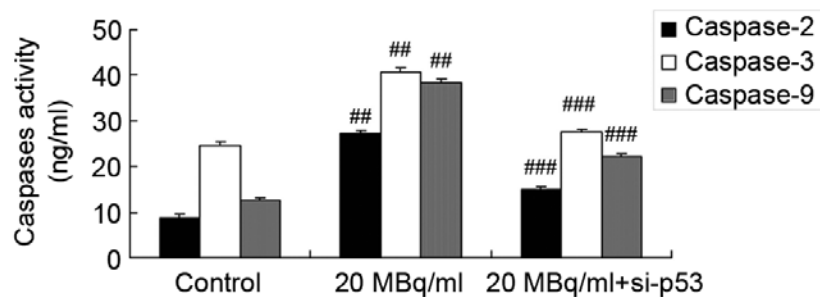


Figure 6. si-p53 inhibits the effects of iodine-131 on caspase-2, -3 and -9 expression levels in human cardiac muscle cells. ## $p < 0.01$ vs. the control group, ### $p < 0.01$ vs. 20 MBq/ml iodine-131 group.

si-p53 inhibits the effects of iodine-131 on the regulation of Bax/cytochrome c/caspase-3 signaling pathway in human

cardiac muscle cells. We investigated whether p53 is involved in the effects of iodine-131 on apoptosis. Figs. 5B-D and 6

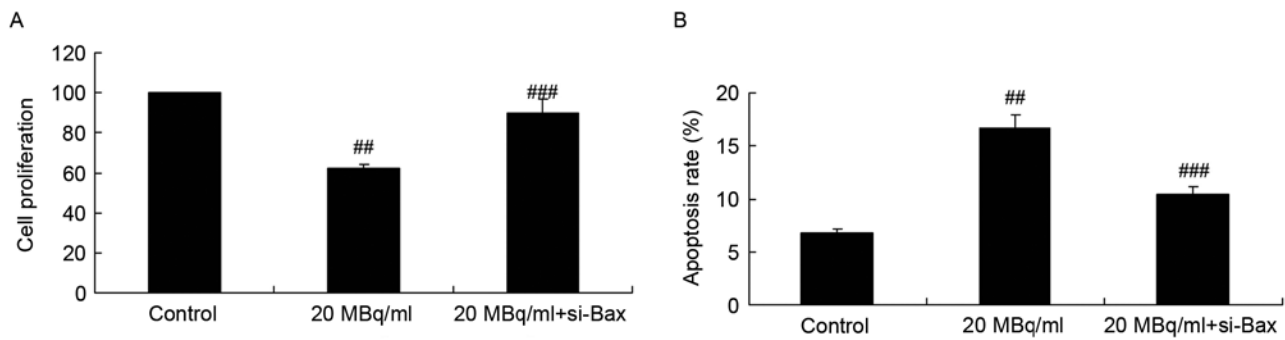


Figure 7. si-Bax reduced the effects of iodine-131 on cell proliferation and apoptosis in human cardiac muscle cells. si-Bax reduced the effects of iodine-131 on cell proliferation (A) and apoptosis (B) in human cardiac muscle cells. ## $p < 0.01$ vs. the control group, ### $p < 0.01$ vs. 20 MBq/ml of iodine-131 group.

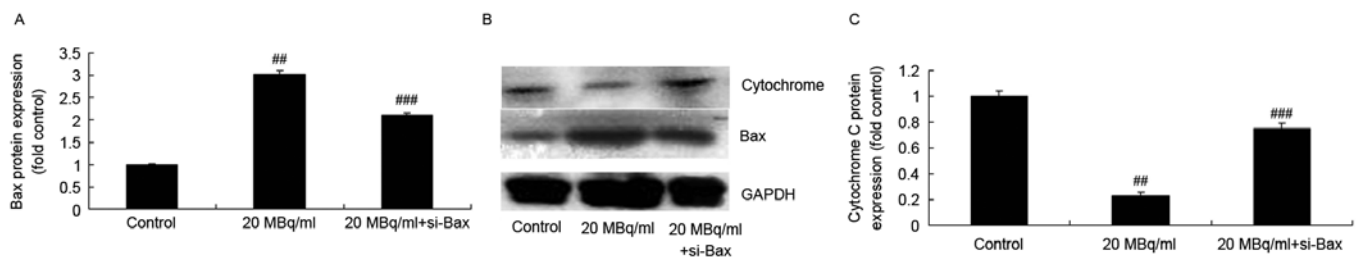


Figure 8. si-Bax reduces the effects of iodine-131 on the Bax and cytochrome *c* signaling pathway in human cardiac muscle cells. Bax and cytochrome *c* protein expression as detected using western blot analysis (B), and statistical analysis (A and C) in human cardiac muscle cells. ## $p < 0.01$ vs. the control group, ### $p < 0.01$ vs. 20 MBq/ml iodine-131 group.

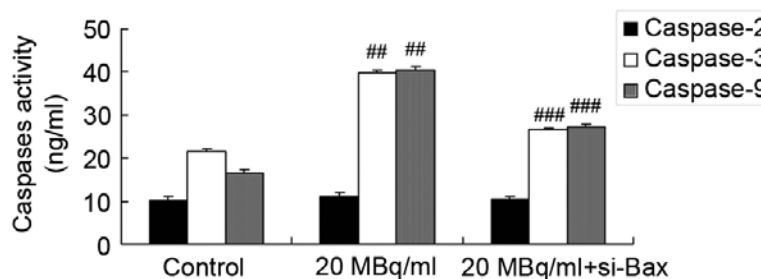


Figure 9. si-Bax reduces the effects of iodine-131 on caspase-2, -3 and -9 expression levels in human cardiac muscle cells. ## $p < 0.01$ vs. the control group, ### $p < 0.01$ vs. 20 MBq/ml iodine-131 group.

show that si-p53 significantly inhibited Bax protein expression and caspase-3/-9 expression levels, and induced cytochrome *c* protein expression in human cardiac muscle cells treated with 20 MBq/ml of iodine-131.

si-Bax reduces the effects of iodine-131 on cell proliferation and apoptosis in human cardiac muscle cells. Next, we investigated the function of si-Bax in the effects of iodine-131 on apoptosis. si-Bax was used to inhibit Bax expression in human cardiac muscle cells treated with iodine-131. As shown in Fig. 7, si-Bax significantly increased the cell proliferation and reduced the apoptosis of human cardiac muscle cells treated with 20 MBq/ml of iodine-131.

*si-Bax reduces the effects of iodine-131 on the cytochrome *c*/caspase-3 signaling pathway in human cardiac muscle cell.* To determine whether si-Bax has a function in the effects of iodine-131 on apoptosis, the cytochrome *c*/caspase-3

signaling pathway in human cardiac muscle cells was analyzed. As shown in Figs. 8 and 9, si-Bax significantly inhibited Bax protein expression and caspase-3/-9 activities, and promoted cytochrome *c* protein expression in human cardiac muscle cells treated with 20 MBq/ml of iodine-131. Yet, si-Bax did not affect caspase-2 expression levels in human cardiac muscle cells following treatment with 20 MBq/ml of iodine-131 (Fig. 9).

si-caspase-2 weakens the effects of iodine-131 on cell proliferation and apoptosis in human cardiac muscle cells. In order to investigate the function of caspase-2 in the effects of iodine-131 on apoptosis and cell proliferation in human cardiac muscle cells, expression of caspase-2 was assessed. Fig. 10A and B indicates that si-caspase-2 significantly increased cell proliferation and reduced apoptosis of human cardiac muscle cells following treatment with 20 MBq/ml of iodine-131. Meanwhile, si-caspase-2 also significantly

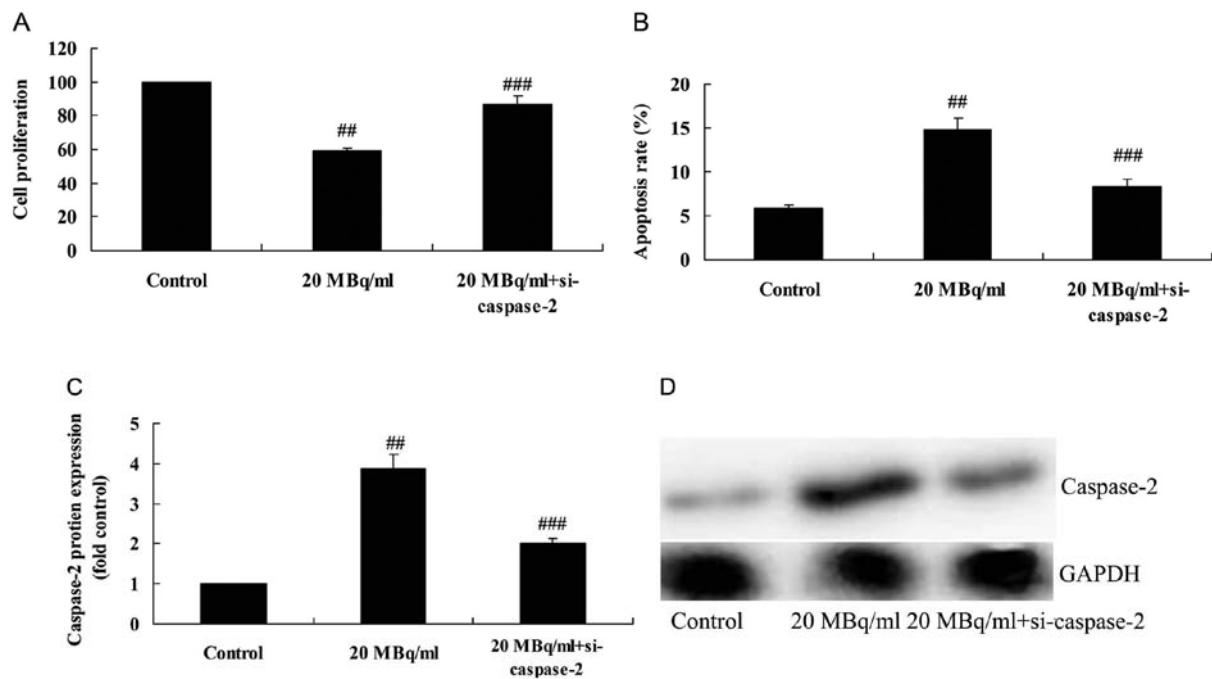


Figure 10. si-caspase-2 reduces the effects of iodine-131 on cell proliferation and apoptosis in human cardiac muscle cells. si-caspase-2 reduced the effects of iodine-131 on cell proliferation (A) and apoptosis (B), and caspase-2 protein expression using western blot analysis (D), and statistical analysis (C) in human cardiac muscle cells. ^{##}p<0.01 vs. the control group, ^{###}p<0.01 vs. 20 MBq/ml iodine-131 group.

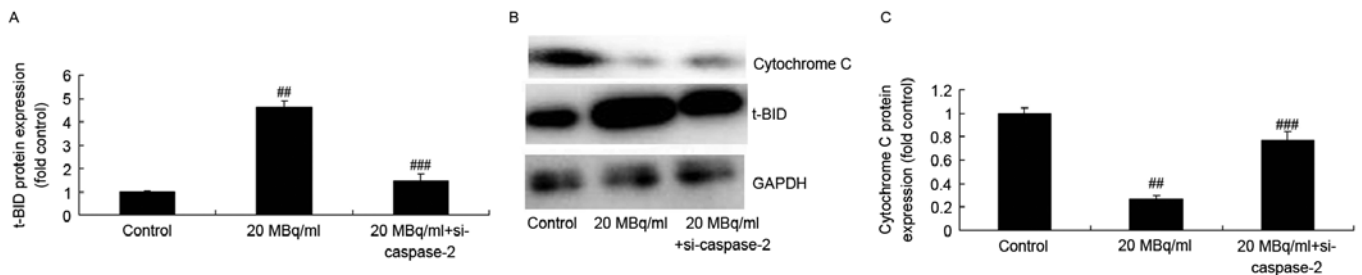


Figure 11. si-caspase-2 weakens the effects of iodine-131 on t-BID/cytochrome *c* protein expression in human cardiac muscle cells. t-BID/cytochrome *c* protein expression using western blot analysis (B), and statistical analysis (A and C) in human cardiac muscle cells. ^{##}p<0.01 vs. the control group, ^{###}p<0.01 vs. 20 MBq/ml iodine-131 group.

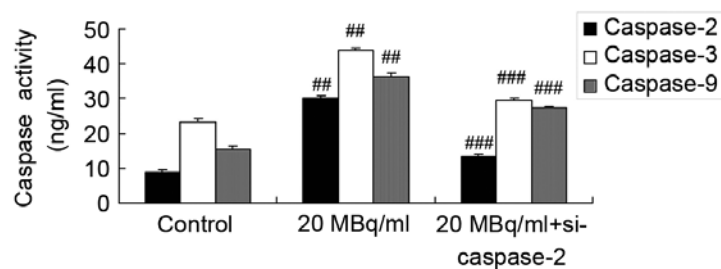


Figure 12. si-caspase-2 inhibits the effects of iodine-131 on caspase-2, -3 and -9 expression levels in human cardiac muscle cells. ^{##}p<0.01 vs. the control group, ^{###}p<0.01 vs. 20 MBq/ml iodine-131 group.

suppressed caspase-2 protein expression of human cardiac muscle cells following treatment with 20 MBq/ml of iodine-131 (Fig. 10C and D).

si-caspase-2 weakens the effects of iodine-131 on the t-BID/cytochrome c/caspase-3 signaling pathway in

human cardiac muscle cells. We determined the function of si-caspase-2 in the effects of iodine-131 on apoptosis. Figs. 11 and 12 indicate that si-caspase-2 inhibited t-BID protein expression and caspase-2/-3/-9 expression levels and promoted cytochrome *c* protein expression in human cardiac muscle cells treated with 20 MBq/ml of iodine-131.

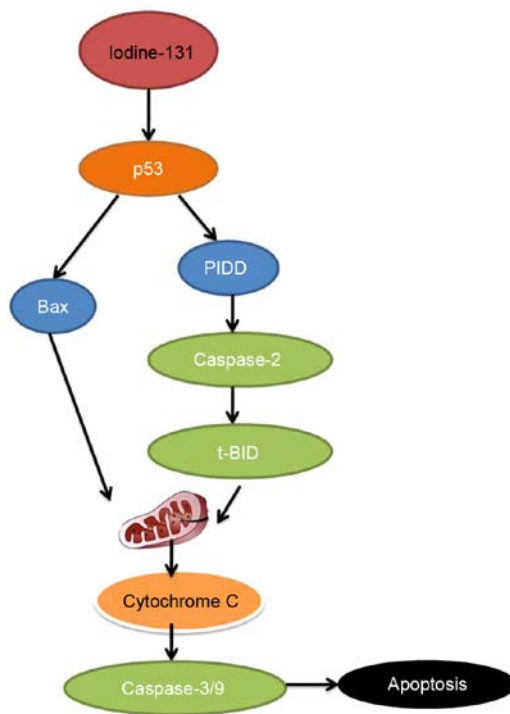


Figure 13. Iodine-131 reduces cell proliferation and induces apoptosis in human cardiac muscle cells through the p53/Bax/caspase-3 and PIDD/caspase-2/t-BID/cytochrome *c*/caspase-3 signaling pathway.

Discussion

Myocardial ischemia reperfusion injury is not only the most common complication of ischemic cardiomyopathy, but also an important reason responsible for insufficiency or failure of the heart and other organs as a result of myocardial impairment after severe trauma (12). As has been reported in the literature, a large degree of myocardial apoptosis is noted in the event of myocardial ischemia reperfusion (13). Therefore, in-depth investigation of the myocardial apoptosis mechanism in myocardial ischemia reperfusion injury is of certain theoretical and practical significance to the prevention and treatment of ischemic cardiomyopathy (13).

It has also been confirmed in human autopsy reports after myocardial infarction that apoptosis does participate in the pathophysiological process of infarction, and the apoptosis process can be further promoted accompanied by the extension of myocardial infarction duration, which thus injures myocardial cells and affects myocardial function (13). p53 is a type of cell cycle regulatory gene that promotes cell apoptosis (14). Caspase-3 is the final executor of cell apoptosis, p53 can directly act on the Bcl-2 regulatory domain, and the p53 protein interacts with the TATAA box binding protein (TBP), which results in inhibition of Bcl-2 gene expression, causing myocardial cell injury (13,14). The results of the present study identified that iodine-131 reduced cell proliferation, induced apoptosis, induced p53, PIDD, t-BID protein expression, suppressed cytochrome *c* protein expression, and increased Bax protein expression, and promoted caspase-2, -3 and -9 expression levels in human cardiac muscle cells.

p53, an important tumor suppressor gene regulating the cell cycle and apoptosis signaling pathway, is closely asso-

ciated with the occurrence of apoptosis. As an important pro-apoptotic factor, p53 promotes cell apoptosis mainly through two pathways, namely, the transcription-dependent and transcription-independent pathways (15). Firstly, p53 can specifically induce expression of cell apoptosis target genes, such as Noxa and Bax, and participates in the endogenous and exogenous apoptosis pathways through these proteins. Secondly, p53 protein allows for transposition from the cytoplasm to mitochondria, and exerts a pro-apoptotic effect by activating the mitochondrial pathway (16). The results of the present study revealed that si-p53 inhibited the effects of iodine-131-reduced cell proliferation and induction of apoptosis in human cardiac muscle cells through regulation of Bax/cytochrome *c*/caspase-3 and PIDD/caspase-2/t-BID/cytochrome *c*/caspase-3 signaling pathway.

The occurrence of cell apoptosis is regulated by intracellular apoptosis-regulating proteins, which can be divided into pro-apoptotic and anti-apoptotic proteins (17). The occurrence of cell apoptosis is attributable to the loss of balance between these two types of mutual antagonistic proteins. Among the established apoptosis-regulating proteins at present, the Bcl-2 family (B-cell leukemia/lymphoma 2-like proteins) plays a crucial role in apoptosis induced by a variety of stimulating signals (17). It is indicated in research that the Bcl-2 and Bax protein levels are directly related to apoptosis regulation; increased Bax promotes cell apoptosis; while increased Bcl-2 inhibits cell apoptosis (18). Consequently, it is proposed that cell survival after stimulation by apoptosis is determined by the Bcl-2/Bax ratio, which can be upregulated in the case of a high Bcl-2 expression level, forms the Bcl-2/Bax heterodimer, inhibiting cell apoptosis. However, the ratio is downregulated in the presence of a high Bax expression level, which forms the Bax/Bax homodimer leading to cell apoptosis (19). In the present study, we found that si-Bax reduced the effects of iodine-131 on cell proliferation and induced apoptosis in human cardiac muscle cells through the cytochrome *c*/caspase-3 signaling pathway.

Bid is a pro-apoptotic protein in the Bcl-2 family proteins, which exists in the cytoplasm in the form of inactive p22Bid. Caspase-8 mediates the hydrolysis of inactive p22Bid in the cytoplasm to produce a major functional fragment p15 (truncated Bid, tBid), together with 2 smaller fragments, p13 and p11 (20). tBid activates the mitochondria to release cytochrome *c* through transposition to the mitochondrial membrane; the latter acts on effector caspases, and finally mediates the apoptosis of multiple cells, including lymphocytes and nerve cells (21). tBid can also be applied in gene therapy, and induces apoptosis of tumor cells or viral-infected cells (22).

Cytochrome *c* can activate the Apaf-1-caspase-9 apoptosis system, and thus activates downstream caspases, including caspase-3, caspase-2, caspase-6, caspase-8 and caspase-10; among these, caspase-8 can cut Bid to form tBid and activates tBid. tBid recruits Bax to embed into the mitochondria after transposition to the mitochondrial membrane and forms the transmembrane channel, which promotes cytochrome *c* to be further released from the mitochondria, forming a positive feedback pathway, and eventually inducing cell apoptosis (23,24).

As a downstream target gene of p53, PIDD is essential in regulating cell apoptosis and stress repair (23). PIDD can bind with RIADD and pro-caspase-2 to form a ternary complex,

which gives rise to caspase-2 activation, thus promoting cell apoptosis through the mitochondrion-dependent pathway (25). The elevated PIDD expression may be an important pathway for the apoptosis induced by the activated p53 pathway (23).

PIDD not only promotes cell apoptosis, but also participates in cell repair under stress (26). Meanwhile, PIDD can directly activate the NF- κ B pathway that can promote DNA injury repair of cells, thus protecting cells from apoptosis. From this point of view, PIDD has certain protective effects on myocardial apoptosis (23). Therefore, PIDD is a gene that possesses dual functions; it can promote cell apoptosis, and prevent myocardial apoptosis by activating the protective signaling pathway of cells.

The caspase family is the initiator and executor of cell apoptosis in mammals, among which, caspase-3 is the most crucial apoptotic protease in the downstream of the caspase cascade reactions (24). Bcl-2 blocks the activation of the upstream caspase protease by interfering with the release of cytochrome *c*, thus inhibiting cell apoptosis (24). As a composition of the ion channel on the mitochondrial membrane, Bax protein allows cytochrome *c* to pass through the mitochondrial membrane, activating caspase-9, and further activating caspase-3, thus resulting in cell apoptosis (9). The results revealed that si-caspase-2 also reduced the effects of iodine-131-reduced cell proliferation and induced apoptosis in human cardiac muscle cells through the t-BID/cytochrome *c*/caspase-3 signaling pathway.

In conclusion, the present study demonstrated that iodine-131 reduced cell proliferation and induced apoptosis of human cardiac muscle cells through the p53/Bax/caspase-3 and PIDD/caspase-2/t-BID/cytochrome *c*/caspase-3 signaling pathway (Fig. 13). Iodine-131 with its effects on the apoptosis of human cardiac muscle cells may prove to be of potential therapeutic significance for designing novel strategies to improve efficacy in myocardial disease.

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