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The protective effects of CHIR99021 against oxidative injury in LO2 cells

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Hepatic ischemia-reperfusion injury is one of the most important factors for the prognosis of liver transplantation and hepatic surgery. It was reported that glycogen synthase kinase-3 (GSK-3) regulated injury response during ischemia-reperfusion. In this study, we investigated the protective effects of the GSK-3 inhibitor CHIR99021 against hepatic ischemia-reperfusion injury. A H_2O_2-induced oxidative injury model using LO2 cells was established. LO2 cells were divided into four groups, including blank control group, CHIR99021 control group treated with CHIR99021 alone, H_2O_2-injury group treated with H_2O_2 and protection group treated with H_2O_2 plus CHIR99021. Cell viability, cell apoptosis or necrosis was determined. Meanwhile, mitochondrial membrane potential, lipid peroxidation, cellular ROS levels, SOD activity, and serum contents of ALS and AST were measured. Protein and mRNA expressions were also detected. The results showed that a cell oxidative injury model was established by treating LO2 cells with 200 μmol/L H_2O_2 for 6 h. Cells exposed to H_2O_2 resulted in a significant decrease of cell viability and increase of cell apoptosis, which was accompanied by increasing ROS levels, disruption of mitochondrial membrane potential, excessive lipid peroxidation, reduction of SOD activity, and increased levels of ALT and AST. Treatment with CHIR99021 significantly protected LO2 cells against H_2O_2-induce oxidative injury by inhibiting the changes of above oxidative injury related indicators. Moreover, CHIR99021 treatment significantly reversed H_2O_2-induced decrease in p-GSK-3β^{Ser9}, Bcl-2, Bcl-xl, survivin and β-catenin expression, whereas it significantly attenuated H_2O_2-induced increase in caspase-3, cleaved caspase-3 and p-JNK protein expression. In conclusion, CHIR99021 protected LO2 cells against H_2O_2-induced oxidative injury through reducing GSK-3β activity and apoptosis, with underlying mechanisms involved in stabilizing mitochondrial membrane potential, attenuating cellular ROS generation, suppressing mitochondria-mediated apoptotic pathway, and activation of GSK-3β/β-catenin signaling pathway.

1. Introduction

Hepatic ischemia-reperfusion injury (HIRI) is a common pathophysiologic process during liver transplantation and surgeries that require blockage of hepatic portal blood flow (Jiang et al. 2005). HIRI is not only the most common causes for liver dysfunction in the early stage after liver transplantation, but also an important factor affecting the long-term survival of liver transplantation patients (Totsuka et al. 2004). The pathogenesis of HIRI is complicated. Excessive oxygen radical generation during ischemia-reperfusion is considered as one of the main reasons for cell death and thus hepatic injury (Petrosillo et al. 2004; Urakami et al. 2007; Zhong et al. 1989). Under healthy conditions, the human body keeps a dynamic balance of oxygen radical levels via precise regulation of its generation and elimination. However, when a liver suffers from ischemia-reperfusion, oxygen radical generation derived from xanthine oxidase system, phagocyte system and mitochondrial respiratory chain is activated, whereas endogenous antioxidants such as SOD are inactivated or exhausted. Hence, it breaks the balance of oxygen radical generation and elimination, and thus results in a sharp increase of oxygen radical in a short time to induce oxidative stress (Anderson et al. 2005; Clavien et al. 1992; Shirasugi et al. 1997). Oxidative stress leads to hepatocyte cytoplasmic Ca^{2+} overload, increasing NO generation, activation of caspase, upregulation of cytokine gene expression and DNA damage, which are involved in the occurrence of apoptosis (Kasahara et al. 1997; Koningsberger et al. 2009; Wedi et al. 1999). During the ischemia-reperfusion, the formation of specific permeability transition pore in mitochondrial inner membrane was significantly increased by the elevation of hepatocyte Ca^{2+} concentration, excessive oxygen radical generation and increase of inorganic phosphate ion. Hence, mitochondrial inner membrane losses its barrier function to result in a large number of solute molecules into mitochondria, leading to mitochondrial oxidative phosphorylation uncoupling, matrix swelling, decrease of membrane potential and disruption of mitochondria. Once mitochondrial damage reaches a certain threshold, it ultimately induces cell death (Macouillard-Poulletier de et al. 1998; Scorrano et al. 2002; Waterhouse et al. 2001). Glycogen synthase kinase-3β (GSK-3β) is a serine/threonine kinase, and recent studies suggest that it may play a key role in ischemia-reperfusion injury. Many pro-survival signaling pathways inactivate the activity of GSK-3β via regulating the phosphorylation GSK-3β, to increase the opening threshold of mitochondrial membrane permeability transition pores, participating in the regulation of ischemia-reperfusion injury (Murphy and Steenbergen 2007; Sugden et al. 2008; Tong et al. 2002). The study found that administering the GSK-3 inhibitors SB216763 and SB415286 before ischemia or reperfusion reduced myocardial infarct area (Obame et al. 2008). Moreover, the GSK-3β phosphorylation level was negatively correlated to myocardial infarct areas in myocardial tissue after ischemia-reperfusion of 5 min (Nishihara et al. 2006). These studies suggest that GSK-3β phosphorylation level is one of the decisive factors for cell survival during ischemia-reperfusion, and GSK-3β specific inhibitor may have a protective effects on cells, tissues and organs under ischemia-reperfusion conditions.

This study investigated the protective effects and underlying mechanisms of GSK-3β inhibitor CHIR99021 against H_2O_2-induced LO2 cell oxidative injury.

2. Investigations and results

2.1. H2O2-induced LO2 cell oxidative injury model was established

We used H_2O_2 to treat LO2 cells to establish an oxidative injury model. Firstly, LO2 cells were treated with H_2O_2 at various concentrations for 3, 6 and 12 h, and cell viability was measured (Fig. 1A). Compared with blank control group (without H_2O_2 insult), H_2O_2 (200, 300, 500, 750 and 1000 μmol/L) treatment for 3 h reduced cell viability of LO2 cells in a concentration-dependent manner, which is similar to cells undergoing H_2O_2 treatment for 6 h and 12 h that they also reduced LO2 cell viability in concentration-dependent manners. Moreover, under H_2O_2 treatment at the same concentration, cell viability decreased in a time-dependent manner. Pearson correlation analysis showed that cell viability after treating with H_2O_2 for 3, 6 and 12 h were negatively correlated with concentrations of H_2O_2, with the correlation coefficient of -0.993, -0.955 and -0.819, respectively.

Next, flow cytometry was employed to determine cell apoptosis induced by H_2O_2. As shown in Fig. 1B, in LO2 cells treated with H_2O_2 (100, 200, 300, 500 and 750 μmol/L) for 3 h, cell apoptosis rate significantly increased in concentration-dependent manners comparing with vehicle control group (0 μmol/L H_2O_2) except cells treated with H_2O_2 at a concentration of 100 μmol/L. After cells were treated with H_2O_2 (100, 200, 300, 500 μmol/L) for 6 h and 12 h, cell apoptosis rate similarly increased in concentration-dependent manners, and the differences were statistically significant comparing with vehicle control group ($p<0.05$). Moreover, cells treated with H_2O_2 at 100, 200, 300 and 500μmol/L time-dependently increased cell apoptosis rate.

According to above results, we chose cells treated with H_2O_2 (200 μmol/L) for 6 h as the optimal conditions for a H_2O_2-induced LO2 cell oxidative injury model in the following experiments. We further determined the changes of oxidative stress-related indicators, including mitochondrial membrane potential, MDA, ROS, SOD activity and levels of ALT and AST. As shown in Fig. 2, flow cytometry results showed that H_2O_2 significantly reduced the mitochondrial membrane potential and SOD activity compared with the blank control group, whereas it significantly increased MDA, ROS generation and levels of ALT and AST.

2.2. CHIR99021 treatment increased LO2 cell viability, while decreased cell apoptosis

The protective effects of CHIR99021 on cell viability were assessed by CCK8. As shown in Fig. 3A, cells treated with CHIR99021 alone at concentrations of 3, 6 and 10 μmol/L did not significantly affect cell viability, compared with the blank control group ($p>0.05$). However, CHIR99021 treatment concentration-dependently inhibited the decrease of cell viability induced by H_2O_2, and cell viability treated with CHIR99021 at 6 and 10μmol/L was significantly higher than that treated with CHIR99021 at 3 μmol/L.

As shown in Fig. 3B, cell apoptosis was not statistically different between the CHIR99021 control group and the blank control group. Apoptosis of LO2 cells challenged by H_2O_2 was effectively reduced by CHIR99021 treatment. After treating with CHIR99021 at concentrations of 6 and 10 μmol/L, H_2O_2-induced cell apoptosis was lower than that of cells treated with 3 μmol/L CHIR99021.

2.3. Effects of CHIR99021 on oxidative stress injury related indicators

JC-1 staining was used to detect mitochondrial membrane potential. As shown in Fig. 4A, CHIR99021 treatment significantly inhibited the disruption of mitochondrial membrane potential induced by H_2O_2, comparing with cells exposed to H_2O_2 alone. In parallel, MDA and ROS levels in the CHIR99021 protection group was significantly lower than that of the oxidative injury group (Fig. 4B and C), while SOD activity in the CHIR99021 protection group was significantly higher than that of the oxidative injury group. Moreover, ALT and AST contents in CHIR99021 protection group were significantly lower than that of oxidative injury group (Fig. 4E). All these indicators were not statistically different between blank control group and CHIR99021 control group.

2.4. CHIR99021 treatment increased p-GSK-3βSer9, Bcl-2, Bcl-xl, surviving and β-catenin expression, while reduced caspase-3, cleaved caspase-3 and p-JNK expression

As shown in Fig. 5A, western blot results showed that there was no significant difference among all four experimental groups. However, p-GSK-3β^{Ser9} protein expression was significantly enhanced in the CHIR99021 protection group, compared with the oxidative injury group. Western blot results also showed that Bcl-2, Bcl-xl, caspase 3 and cleaved caspase 3 protein expression was not significantly different between blank control group and CHIR99021 control group (Fig. 5B). CHIR99021 protection group increased Bcl-2 and Bcl-xl protein expressions and decreased caspase 3 and cleaved caspase 3 protein expressions, compared with the oxidative injury group (Fig. 5B). Similarly, JNK protein expression was not significantly different among all four experimental groups, whereas the CHIR99021 protection group showed lower levels of p-JNK protein expression than that of oxidative injury group (Fig. 5C). Protein expression of β-catenin in both CHIR99021 control group and CHIR99021 protection group was significantly enhanced, and β-catenin protein expression in CHIR99021 protection group was higher than that of the CHIR99021 control group (Fig. 5C).

Fluorescence quantitative PCR was carried out to detect Bcl-2, Bcl-xl, β-catenin, survivin and caspase 3 mRNA expression. QPCR results were standardized by setting gene expression in the blank control group as reference and calculating the ratio of all groups through dividing by the value of blank control group. As shown in Fig. 5D, Bcl-2, Bcl-xl, surviving and caspase 3 mRNA expression in CHIR99021 control group were not significantly different from the blank control group. Whereas β-catenin mRNA expression in CHIR99021 control group was 1.3 times higher than that in the blank control group, and the difference was statistically significant ($p<0.05$). Bcl-2, Bcl-xl, β-catenin and survivin mRNA expressions increased in the CHIR99021 protection group, whereas caspase-3 mRNA expression decreased in the CHIR99021 protection group, comparing with oxidative injury group ($p<0.05$).

3. Discussion

3.1. LO2 cell oxidative injury model

A number of studies reported that a cell oxidative injury model was induced by H_2O_2. However, the concentration of H_2O_2 and treating time were quite different in these studies, displaying that cell apoptosis was induced by H_2O_2 at minimum concentration of 50 μmol/ L for 8 h (Pantopoulos et al. 1996), and at maximum concentration of 1000 mmol/L for 20 min (Lou et al. 2013). In our study, we determined cell viability and apoptosis through exposing LO2 cells with H_2O_2 at concentrations of 200, 300, 500, 750 and 1000 μmol/L for 3, 6 and 12 h, respectively, and found that the H_2O_2-induced cell viability decreased in both concentration- and time-dependent manners, suggesting that H_2O_2 insult could induce LO2 cell damage.

At present, cell oxidative injury is induced by oxidative stress or hypoxia/reoxygenation in vitro, and the cell viability usually decreases by 30 ~ 50%. When using H_2O_2 insult to produce oxidative injury model, if H_2O_2 concentration is too low, it might result in a similar effect as hypoxia-ischemia/ischemic preconditioning, increasing cell tolerance against ROS. However, if cells suffer from much too serious injury, cell necrosis rate is too high to dramatically reduce the number of cells with relatively reversible damage. On the other hand, it may induce cell apoptosis/necrosis too quickly or high proportions of cells with direct mechanical injury. Hence, these subsequently lead to adverse effects on the research of protective agents or approaches (Altenhofer et al. 2015; Hernandes et al. 2014; Hsu et al. 2014). In addition, the minimum interval for the evaluation of gene expression and the corresponding protein products is 4 h in order to facilitate the measure of gene and

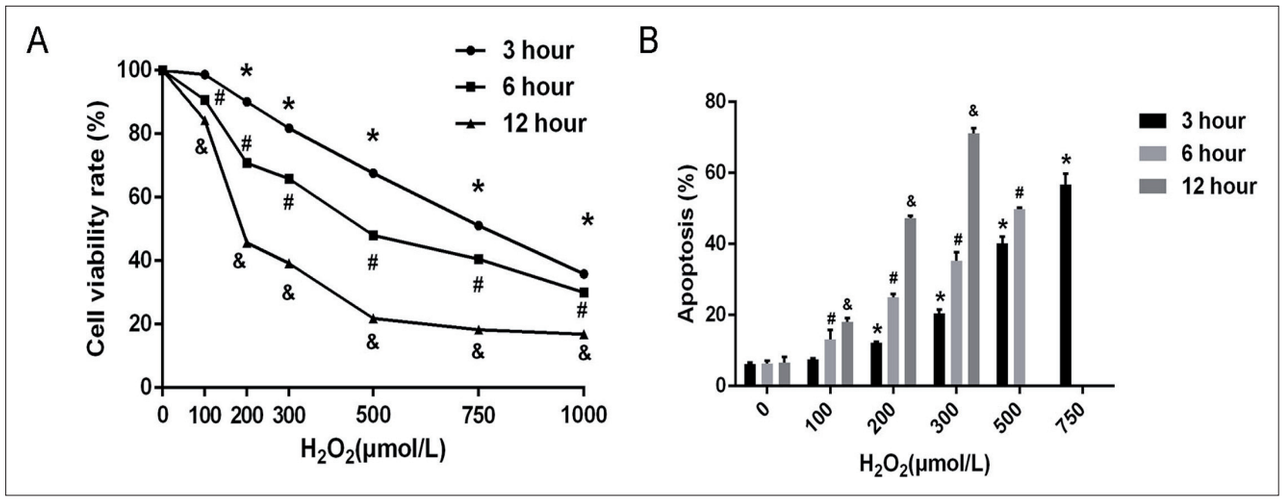

Fig. 1: Effects of H_2O_2 on survival and apoptosis of LO2 cells at different concentrations and timepoints. A, H_2O_2 induced decrease of cell viability in concentration- and time-dependent manners. B, H_2O_2 induced increase of cell apoptosis in concentration- and time-dependent manners. *, #, &, versus blank control group in indicated timepoints, respectively, $p<0.05$.

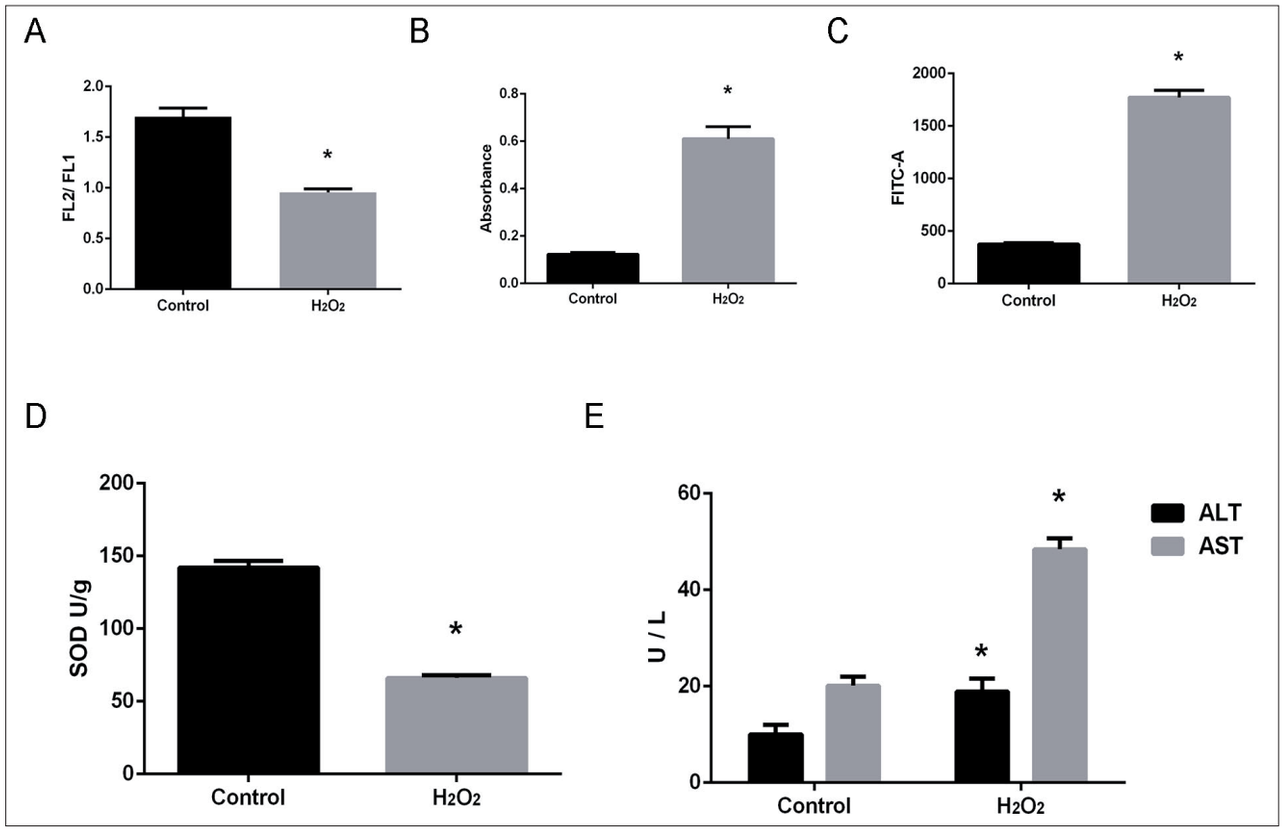

Fig. 2: Changes of oxidative stress injury related indicators in LO2 cells undergoing treatment of H_2O_2 (200μmol/L, 6 h). A, FL2/FL1 fluorescence intensity ratio was used to evaluate the change of mitochondrial membrane potential. The FL2/FL1 ratio in the H_2O_2-injury group was lower than that of the blank control group. B: The level of lipid peroxidation in the H_2O_2-injury group was higher than that of the blank control group. C: The level of intracellular ROS in the H_2O_2-injury group was higher than that of the blank control group. D: Compared with blank control group, the SOD activity decreased in the H_2O_2-injury group. E: The contents of both ALT and AST of culture medium in the H_2O_2-injury group were higher than those in the blank control group. * versus the blank control group, $p < 0.05$.

protein expression of injury cells induced by H_2O_2 and under the treatment of protective agents.

In this study, results of cell viability and apoptosis detection showed that H_2O_2 exposure induced LO2 cell oxidative injury. Especially, H_2O_2 exposure with a concentration of 200 μmol/L for 6 h, not only reduced cell viability, but also significantly increased cell apoptosis, accompanied by increase of ROS, disruption of mitochondrial membrane potential, excessive lipid peroxidation and reduction of SOD activity. The changes of all these indicators are consistent with the responses of tissue suffering ischemia-reperfusion or cellular oxidative stress, with good stability and repeatability, indicating that LO2 cells oxidative injury model induced by treating with H_2O_2 (200 μmol/L) for 6 h can be applied in following experiments.

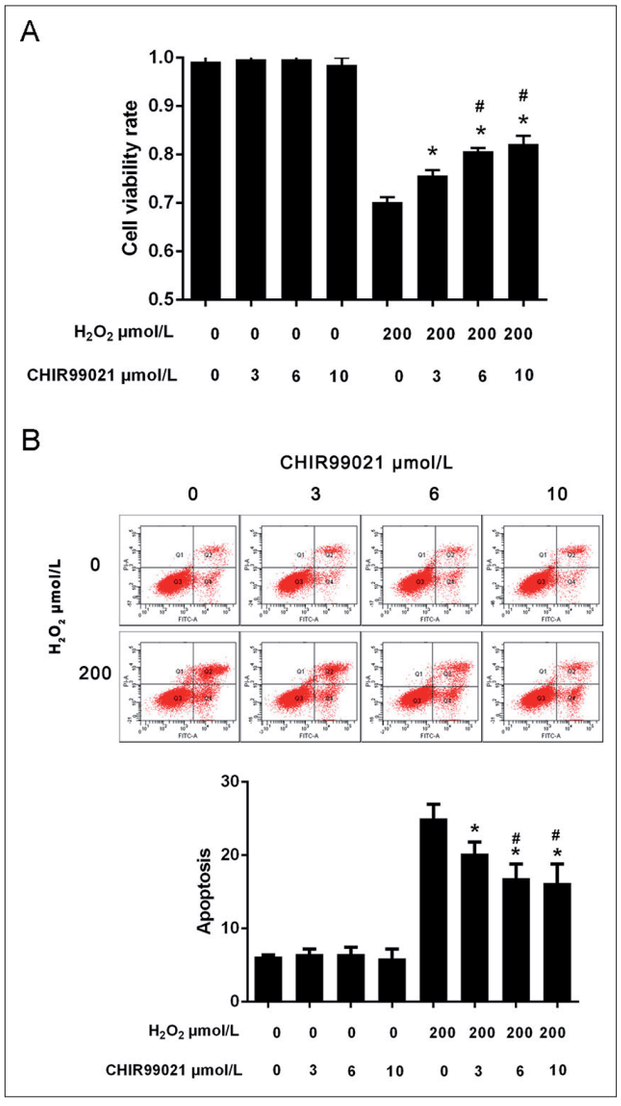

Fig. 3: CHIR99021 alleviated oxidative injury in LO2 cells. A, cell viability in CHIR99021 control group had no significant difference with that in the blank control group, while cell viability in the protection group was significantly higher than that of the injury group. B: cell apoptosis in CHIR99021 control group had no significant difference with that in the blank control group, while cell apoptosis in protection group significantly decreased, comparing with that of H_2O_2-injury group. *, versus H2O2-injury group, $p<0.05$. #, versus protection group (3 mol/L), $p<0.05$.

3.2. GSK-3β small-molecular inhibitor CHIR99021 protected LO2 cell against oxidative injury

GSK-3 is a serine/threonine protein kinase with intrinsic activity, which is regulated by phosphorylation and intracellular location. It has two highly homologous subtypes: GSK-3α and GSK-3β. In addition to regulating glycogen metabolism, GSK-3β also participates in the regulation of many physiological and pathological events in liver, such as apoptosis, proliferation, differentiation and oxidative stress. (Thompson and Monga 2007). Studies have shown that inhibiting the activity of GSK-3β can stabilize the mitochondrial function in the process of ischemia-reperfusion, maintaining the energy metabolism of liver, and reducing the release of local inflammatory factors (Ren et al. 2011; Varela et al. 2010).

In this study, results of CCK8 assay and apoptosis detection with flow cytometry showed that treatment with GSK-3β inhibitor CHIR99021 did not significantly affect cell viability and apoptosis in the CHIR99021 control group, comparing with that in the blank control group. Whereas, CHIR99021 treatment in the protection group significantly increased cell viability and decreased cell apoptosis, compared with the oxidative injury group. Comparison of the three concentrations of CHIR99021 indicated that the difference of protective effects between 3 and 6 μmol/L CHIR99021 treatment groups were statistically significant, while the difference between 6 and 10 μmol/L CHIR99021 treatment groups was not significant, suggesting that a better cell protective effect against H_2O_2-induced oxidative injury could be obtained by treatment with 6μmol/L CHIR99021. Then we used this treatment condition to determine the indicated oxidative stress related indexes, including mitochondrial membrane potential, ROS, lipid peroxidation, SOD activity, and so on. We found that treatment with CHIR99021 alone did not significantly affect the levels of all the indicated indicators, comparing with the blank control group. However, compared with the oxidative injury group, CHIR99021 treatment in the protection group significantly inhibited the elevations of ALT/AST, reduced the lipid peroxidation and the consumption of SOD. Besides, it also attenuated the alteration of mitochondrial membrane potential by approximately 25%, and reduced intracellular ROS generation by approximately 50%. These findings indicated that CHIR99021 (6μmol/L) treatment inhibited oxygen radical generation, attenuated cell lipid oxidation and consumption of antioxidant enzyme, to protect against H_2O_2-induced cell injury and mitochondrial dysfunction.

The specific mechanisms involved in cellular oxidative damage induced by GSK-3β are complicated and not yet fully understood. One of the mechanisms is that the activation of GSK-3β is involved in the disintegration of mitochondrial membrane potential and the releasing of apoptotic protein to induce intrinsic apoptosis (King et al. 2001; Linseman et al. 2004; Rottmann et al. 2005). In this study, phosphorylation of p-GSK-3β^{Ser9} in the protection group increased significantly, indicating that CHIR99021 significantly inhibited the activity of GSK-3β. Furthermore, as a key protease related with apoptosis, a significant decrease in the expression total caspase-3 and cleaved caspase-3, suggests that CHIR99021 regulates cell apoptotic signaling through inhibition of GSK-3β.

The JNK signaling pathway is one of the most important signaling pathways to regulate mitochondria-mediated apoptotic signaling pathway. In the process of oxidative injury, ROS positively regulates the activation of several signaling molecules, such as apoptotic signal-regulating kinase 1 (ASK1), mixed-lineage kinase 3 (MLK3) and Src protein (Hong and Kim 2007; Kai et al. 2001; Nagai et al. 2007). On the other hand, ROS indirectly activates JNK or maintains sustained activation of JNK by inhibiting the activation of JNK inhibitors, such as mitogen-activated protein kinase phosphatase (kinase phosphatases MAP, MKPs) (Kamata et al. 2005). Previous studies have confirmed that JNK is activated in hepatic ischemia-reperfusion injury, and a JNK inhibitor can reduce the level of apoptosis (Uehara et al. 2005). In this study, we observed that the phosphorylation of JNK in the CHIR99021 protection group was significantly reduced; indirectly confirming that CHIR99021 inhibited GSK-3β and thus reduced apoptosis mediated by mitochondrial signaling pathway.

β-Catenin is a key signal molecule in the Wnt signaling pathway, and its activity regulation is accomplished by GSK-3 mediated phosphorylation. Previous study of cardiomyocytes and neurons indicated administration of GSK-3 inhibitors SB217632 before ischemia or reperfusion increased intracellular β-catenin accumulation and thus activated the GSK-3β/β-catenin signaling pathway, further enhancing the expression of Bcl-2 and survivin to exert the effects of anti-apoptosis (Obame et al. 2008). The study of myocardial ischemic preconditioning in rats showed that β-catenin knockout eliminated the protective effects of ischemic preconditioning on myocardial ischemia. Bcl-2 and surviving played an important role in this process. In the present study, compared with the injury group, the CHIR99021 protection group showed a significant accumulation of intracellular β-catenin, accompanied by increased expression of Bcl-2 and Bcl-x1. In summary, CHIR99021 protected LO2 cells against oxidative injury, the protective mechanism might be involved in restoring the stabilization of mitochondrial membrane potential, reducing intracellular generation of ROS, inhibiting mitochondrial apoptotic signaling pathway, and enhancing the activation of GSK-3β/β-catenin signaling pathways.

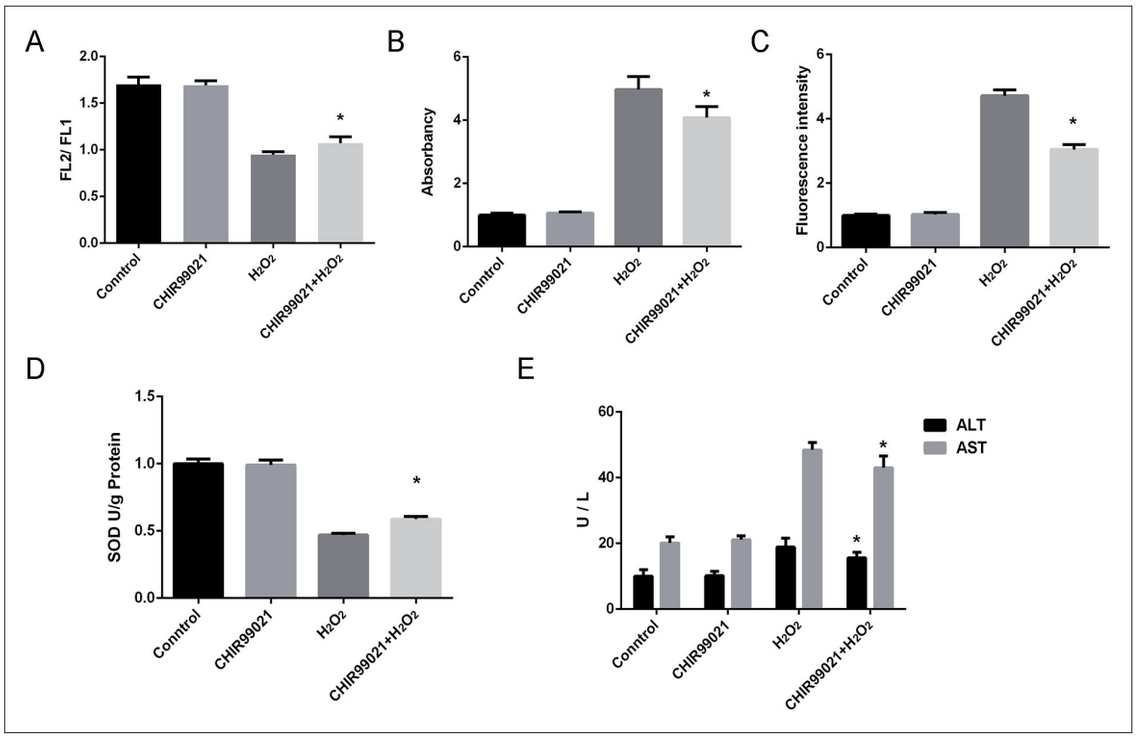

Fig. 4: Effects of CHIR99021 on oxidative injury related indicators in LO2 cells. All the oxidative stress injury related indexes had no significant difference between the blank control group and the CHIR99021 control group. Compared with the injury group, mitochondrial membrane potential was significantly restored in the protection group (A). The level of lipid peroxidation in the protection group was significantly lower than that of the injury group (B). The intracellular ROS was significantly decreased in the protection group (C). The activity of SOD in the protection group was significantly greater than in the injury group (D). The contents of ALT and AST was significantly decreased in the protection group (E). *, versus injury group, $p < 0.05$.

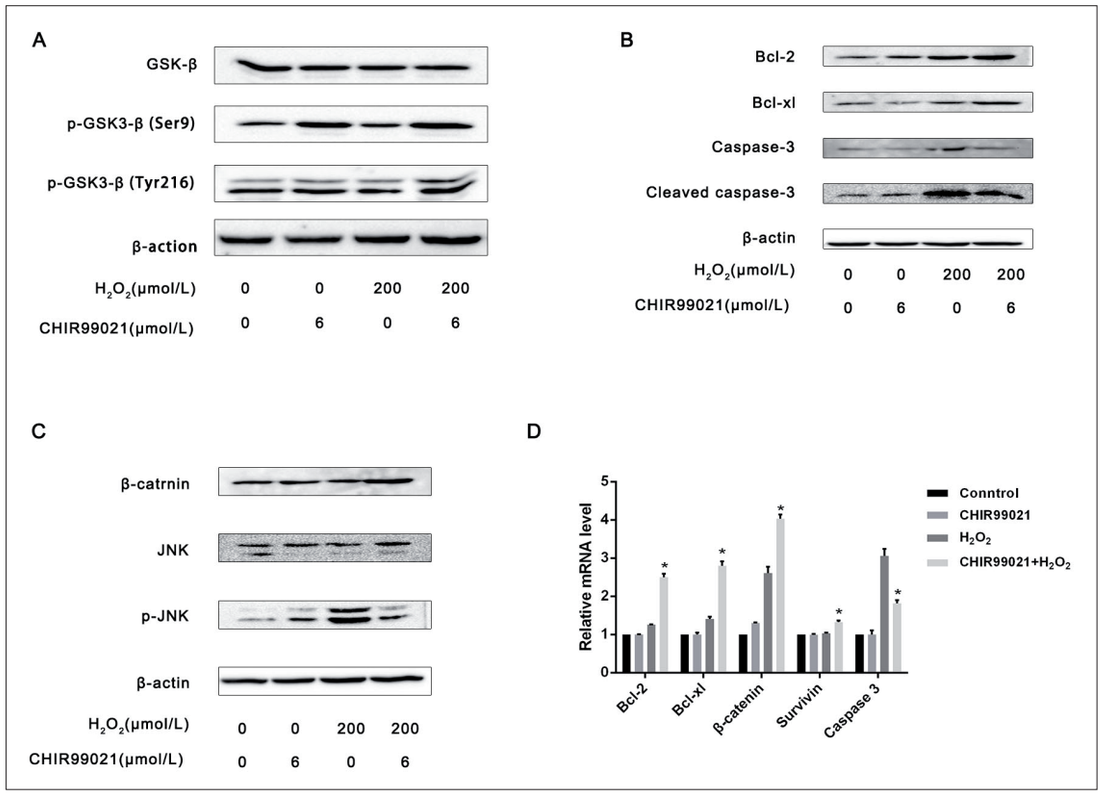

Fig. 5: The expression of indicated protein was determined by western blot or qPCR. All the indicated protein expressions had no significant difference between the blank control group and the CHIR99021 control group. P-GSK-3β^{Ser9} in the protection group was significantly greater than that of injury group (A). Bcl-2 and BCL xl expresion increased, while caspase-3 and cleaved caspase-3 expression significantly decreased in the protection group, compared with the H_2O_2-injury group (B). β-Catenin expression in the protection group increased, whicle p-JNK expression was significantly decreased in the protection group, compared with the H_2O_2-injury group (C). QPCR results showed that Bcl-2, BLC xl, β-catenin and surviving mRNA expression determined increase, while caspase-3 mRNA expression decreased in the protection group, compared with the H_2O_2-injury group.

4. Experimental

4.1. Cell culture

Human normal LO2 hepatic cells were provided by the pharmacological laboratory of Sun Yat-sen University. LO2 cells were cultured in RPMI-1640 medium with 10% fetal bovine serum at 37 °C under a humidified atmosphere of 5% CO_2.

4.2. Experimental design

To establish an oxidative injury cell model, LO2 cells were treated with H_2O_2 in concentrations of 0, 100, 200, 300, 500, 750 and 1000 μmol/L for 3, 6 and 12 h, respectively, to determine cell viability. For apoptosis assay, LO2 cells were treated with H_2O_2 in concentrations of 0, 100, 200, 300, 500 and 700μmol/L for 3 h, of 0, 100, 200, 300, 500 μmol/L for 6 h, and of 0, 100, 200 and 300 μmol/L for 12 h. When measuring the effects of CHIR99021, LO2 cells were allocated into 4 groups, including blank control group, CHIR99021 control group (treated with CHIR99021 alone), oxidative injury group (treated with 200 μmol/L H_2O_2 alone), and CHIR99021 protection group (treated with 200 μmol/L H_2O_2 and CHIR99021). In CHIR99021 control group and CHIR99021 protection group, CHIR99021 (BioVision, CA, USA) was used with concentrations of 3, 6 and 10 μmol/L for cell viability and apoptosis assays, while CHIR99021 at the concentration of 6 μmol/L was used in experiments to detect oxidative injury indexes.

4.3. CCK8 assay to detect cell viability

Cells were seeded into 96-well culture plate at the density of 5×10^3/well and treated with above indicated conditions of H_2O_2 and/or CHIR99021. CCK8 assay was carried out according to product instruction (Dojindo, Kumamoto, Japan). Briefly, 96-well culture plate was added 10μl CCK-8 solution each well, and incubated in incubator for additional 1 h. The absorbance of each well was measured by microplate reader at 450 nm.

4.4. Flow cytometric analysis to detect apoptosis with annexin V-FITC / PI double staining

Cells were seeded into 6-well culture plate at the density of 4×10^5/well and maintained at 37 °C under a humidified atmosphere of 5% CO_2 for 24 h. Then the complete culture medium was replaced with serum free medium. Cells were treated with H_2O_2 and/or CHIR99021 at indicated concentrations. After incubation for the indicated time, annexin V-FITC cell apoptosis assay was carried out according to product instructions (Keygen Biotech, Nanjing, China). When treatment was finished, cells were detached using trypsin solution with EDTA and collected. Then cells were washed with PBS and re-suspended with 500 μl binding buffer. Suspending cells were mixed with 5 μl annexin V-FITC and 5 μl propidium iodide, successively. After incubating at room temperature in the dark for 15 min, apoptosis of cells were analyzed by flow cytometry (Beckman Coulter, FL, USA).

4.5. Mitochondrial membrane potential assay

Cells were seeded into 6-well culture plate at the density of 4×10^5/well and maintained at 37 °C under a humidified atmosphere of 5% CO_2 for 24 h. Cells were treated with H_2O_2 and/or CHIR99021 with indicated concentrations and incubated for additional 6 h. mitochondrial membrane potential was detected according to manufacturer's instructions. Briefly, 10^6 cells were made a suspended solution. After washed using PBS, cells were centrifuged and collected. Then cells were re-suspended using 500 μl prepared JC-10 working solution, incubated at 37 °C for 15 min, and centrifuged to collect cells, following washing with 1×incubation buffer and re-suspending in 500μl 1×incubation buffer. Finally, cells were analyzed using flow cytometry (Beckman Coulter, FL, USA). Mitochondrial membrane potential was expressed as ratio of FL2/FL1 fluorescence intensity.

4.6. Lipid peroxidation (MDA) detection

Cells were treated with H_2O_2 and/or CHIR99021 at indicated concentrations and incubated for additional 6 h. MDA measure was carried out following the MDA detection kit's instructions (Keygen Biotech, Nanjing, China). The absorbance of each sample was measured using microplate reader at 532 nm. Data were expressed using the absorbance value at 532 nm to represent the content of MDA.

4.7. Cellular ROS detection

Cells were treated with corresponding treatments, and then incubated for additional 6 h. Then cells were detached and collected with trypsin solution without EDTA. After washed with PBS, cells were collected and suspended in 1640 medium with 10 μmol/L DCFH-DA. Then it was incubated at 37 °C for 40 min. During the incubation, cells were mixed every 3-5 min to make the DCFH-DA probe well contact with cells. Cells were washed with RPMI-1640 medium for 2 times to remove extracellular DCFH-DA. Mean fluorescence intensity of FITC-A was measured using flow cytometry.

4.8. Cellular superoxide dismutase (SOD) detection

Cells were treated with H_2O_2 and/or CHIR99021 with indicated concentrations and incubated for additional 6 h. SOD activity was measured and calculated according to SOD detection kit's instruction (Keygen Biotech, Nanjing, China).

4.9. Glutamic-pyruvic transaminase (ALT) and glutamic oxalacetic transaminase (AST) detection

Cell culture supernatant was collected. The contents of ALT and AST were measured according to ALT/AST detection kit's instructions (Nanjing Jiancheng Bio-engineering Institute, Nanjing, China). OD value was read using microplate reader at 510 nm. OD value of each sample was subtracted OD value in blank well and calculated the active unit of ALT or AST using standard curve.

4.10. Western blot

Cells were treated with H_2O_2 and/or CHIR99021 with indicated concentrations and incubated for additional 6 h. Then protein extraction was performed. Protein concentration of each sample was determined by the Bradford method and separated by 10% SDS-PAGE electrophoresis. Then protein was transferred to PVDF membrane and incubated with blocking solution for 1 h. After blocking, membrane was incubated with following primary antibody overnight at 4°C: anti-GSK-3β, anti-p-GSK-3β, anti-Bcl-2, anti-Bcl-xl, anti-caspase3, anti-cleaved caspase3, anti-β-catenin, anti-JNK and anti-p-JNK. Then then membrane was incubated with corresponding secondary antibody for 1 h. Bands were developed using chemiluminescence reagent (ECL, Forevergen, Guangzhou, China). Densitometry was carried out using Quantity One software..

4.11. Fluorescence quantitative PCR (qPCR)

Total RNA was extracted from cell sample using Takara reagent (CodeD9108B RNAiso Plus Total RNA). After performed the reverse transcription reaction, SYBR Green fluorescent quantitation PCR kit was used to determine indicated gene expression. qPCR was carried out as following conditions: Initial denaturing at 95°C for 30 s, 40 cycles PCR reaction performing at 95°C for 10 s, 60°C for 20 s, and 72°C for 30 s. Analysis of dissolution curve was performed as follows: 95°C for 5s, 65°C for 60s°C 97°C for 0s. GAPDH was used as the reference gene. The relative gene expression levels were calculated using $2^{-\Delta\Delta Ct}$ method. The primers sequences are shown in the Table.

Table: Primers sequences for quantitative RT-PCR

	Forward (5'-3')	Reverse (5'-3')
Bcl-2	5' TATCCAATCCTGTGCTGCTATC 3'	5' ACTCTGTGAATCCCGTTTGAA 3'
Bcl-xl	5' GAATGACCACCTAGAGCCTTGG 3'	5' TGTTCCCATAGAGTTCCACAAAAG 3'
Caspase3	5' GACTCTGGAATATCCCTGGACAACA 3'	5' CTGAGGTTTGCTGCATCGACA 3'
Survivin	5' AGAACTGGCCCTTCTTGGAGG 3'	5' CTTTTTATGTTCCTCTATGGGGTC 3'
Survivin	5' AGAACTGGCCCTTCTTGGAGG 3'	5' CTTTTTATGTTCCTCTATGGGGTC 3'
β-Catenin	5' GATTTGATGGAGTTGGACATGG 3'	5' TGTTCTTGAGTGAAGGACTGAG 3'
ACTB	5' AAGATGACCCAGATCATGTTTGAG 3'	5' GCAGCTCGTAGCTCTTCTCCAG 3'

4.12. Statistical analysis

Data were analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The comparison between two groups was analyzed using Student t-test. Pearson correlation analysis was used to determine the relationship between cell viability and the concentration of H_2O_2 treatment. $P < 0.05$ was considered statistically significant.

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