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JNK and AKT/GSK3β signaling pathways converge to regulate periodontal ligament cell survival involving XIAP

Mouda Wei [1], Min Zhang [1], Andrew Adams, Yinzhong Duan *

Department of Orthodontics, School of Stomatology, Fourth Military Medical University, Xi'an, Shaanxi, China

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ABSTRACT

Periodontal ligament cells (PDLCs) were incubated with H_2O_2 and the levels of XIAP protein, protein kinase B (AKT), phosphorylated forms of AKT (pAKT), c-Jun N-terminal kinase (JNK), and glycogen synthase kinase-3β (GSK3β) were determined by western immunoblotting or immunocytochemistry. After overexpression and knockdown of XIAP, the AKT, pAKT, JNK and GSK3β levels were determined in PDLCs exposed to H_2O_2.

We demonstrated that 72 h of 250 μM H_2O_2 exposure resulted in an increase in apoptosis. Meanwhile, XIAP levels were decreased with 72 h of 250 μM H_2O_2 exposure, while there were also a decrease of JNK2, AKT, pAKT, and GSK3β levels. Such reductions induced by 72 h of 250 μM H_2O_2 treatment were partially recovered in PDLCs overexpressing XIAP. Interestingly, these reductions (except for pAKT) were mimicked by RNA interference of XIAP. These results suggest that, after 72 h of 250 μM H_2O_2 exposure, Akt, JNK, and GSK3β intracellular kinase signaling pathways converge to regulate PDLC survival involving XIAP.

1. Introduction

The periodontal ligament (PDL) is a soft connective tissue embedded between the cementum (a thin layer of mineralized tissue covering the roots of teeth) and the inner wall of the alveolar bone socket, which helps to constrain teeth within the jaw. Furthermore, the PDL not only has an important role in supporting teeth, but also contributes to tooth nutrition, homoeostasis, and repair of damaged tissue [1–3].

Hydrogen peroxide (H_2O_2) is generated by almost all sources of oxidative stress and can penetrate cellular membranes [4]. Moreover, H_2O_2 is widely used in dental clinics to bleach teeth, enhance gingival healing, and diminish bacterial populations in dental plaque [5]. H_2O_2-induced oxidation of periodontal ligament cells (PDLCs) is inevitable, and the oxidative stress can induce DNA damage and cell death. However, there has been a lack of reports that elucidate the mechanisms of H_2O_2-induced PDLC death including apoptosis.

Kinase signaling pathways play a key role in signal transduction in all cellular processes including apoptosis. Three kinase pathways in particular are important for apoptotic signaling: c-Jun N-terminal kinase (JNK), glycogen synthase kinase-3β (GSK3β), and protein kinase B (AKT) pathways. However, the downstream targets have not been clearly defined, which link these kinases to cell survival, especially XIAP. AKT, also known as protein kinase B1 or RAC kinase, is a member of the phosphatidylinositol 3-OH-kinase-regulated serine/threonine kinases [6–8]. Previous study has shown a possible link between XIAP expression and Akt activity, Veillette and colleagues [9] found that reduced XIAP expression was concomitant to reduced pAkt expression suggesting a possible functional link between XIAP expression and Akt activity. Accumulated evidence shows that AKT and its downstream targets constitute a major cell survival pathway [10,11]. In addition, it has been demonstrated that XIAP is a physiological substrate of AKT that interacts with and phosphorylates XIAP at serine-87. Phosphorylation of XIAP by AKT inhibits both its auto-ubiquitination and cisplatin-induced ubiquitination [12].

In this study, we focus on the role of AKT, JNK2, and GSK3β kinase signaling pathways in H_2O_2-induced PDLCs apoptosis to elucidate the mechanisms by which the relationship between XIAP and kinase signaling pathways acts in PDLCs survival.

2. Materials and methods

PDLs and bones were obtained from extracted human impacted third molars and associated maxillary alveolar bones at the Department of Orthodontics, School of Stomatology, Fourth Military

* Corresponding author. Address: Department of Orthodontics, School of Stomatology, Fourth Military Medical University, 169 Changle West Road, Xi'an, Shaanxi 710032, China. Fax: +86 02984776481.
E-mail address: duanyz@fmmu.edu.cn (Y. Duan).
[1] Mouda Wei and Min Zhang contributed equally to this work.

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Medical University, Xi'an, Shaanxi, China. Release of the cells from the PDLs and maxillary bones was performed as described previously in our lab, cells were passed no more than 20 times. Briefly, the tissues were digested with 3 mg/ml type 1 collagenase (BioBasic, Toronto, Canada) and 4 mg/ml dispase (GIBCO BRL, Grand Island, NY, USA) for 1 h by shaking at 37 °C in a 5% CO_2 incubator to obtain single-cell suspensions. The PDLCs were then cultured in α-minimal essential medium (α-MEM; GIBCO BRL, Grand Island, NY, USA) supplemented with 15% fetal bovine serum (GIBCO BRL, Grand Island, NY, USA), 2 mM L-glutamine (GIBCO BRL, Grand Island, NY, USA), 100 μM L-ascorbate-2-phosphate (Sigma–Aldrich, St. Louis, MO, USA), and 1% antibiotic–antimycotic solution (GIBCO BRL, Grand Island, NY, USA). The cells were incubated at 37 °C with 5% CO_2. Anti-XIAP and anti-GSK3β primary antibodies were purchased from Becton Dickinson Biosciences (San Jose, California, USA). An anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibody was purchased from Ambion (Grand Island, NY, USA) and an anti-JNK2 primary antibody was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). Anti-AKT and anti-phosphorylated AKT (pAKT) antibodies were purchased from Cell Signaling (Danvers, Massachusetts, USA).

2.1. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay to evaluate the cell viability

The MTT assay was performed as described previously [13]. Briefly, cells were seeded at a density of 5×10^3 cells/well in 96-well plates and treated with H_2O_2 at various concentrations (3.9, 7.81, 15.625, 31.25, 62.5, 125, 250, 500, and 1000 μM). After 24 h of treatment, the medium was removed and the cells were incubated with 1 mg/ml MTT (Sigma Chemical Co., USA) for 4 h. Then, the non-metabolized MTT was discarded and 100 μl DMSO was added to each well. The absorbance in each well was measured by a Multiskan MCC/340P plate spectrophotometer at a reading wavelength of 570 nm and a reference wavelength of 690 nm. Each experiment was performed in triplicate.

2.2. Annexin V-FITC/propidium iodide (PI) assay

Apoptosis was measured using an annexin V-FITC/PI apoptosis detection kit (BD PharMingen, San Jose, CA, USA) according to the manufacturer's instructions in reference to published standard settings [14–15]. Annexin V-FITC is used to quantitatively

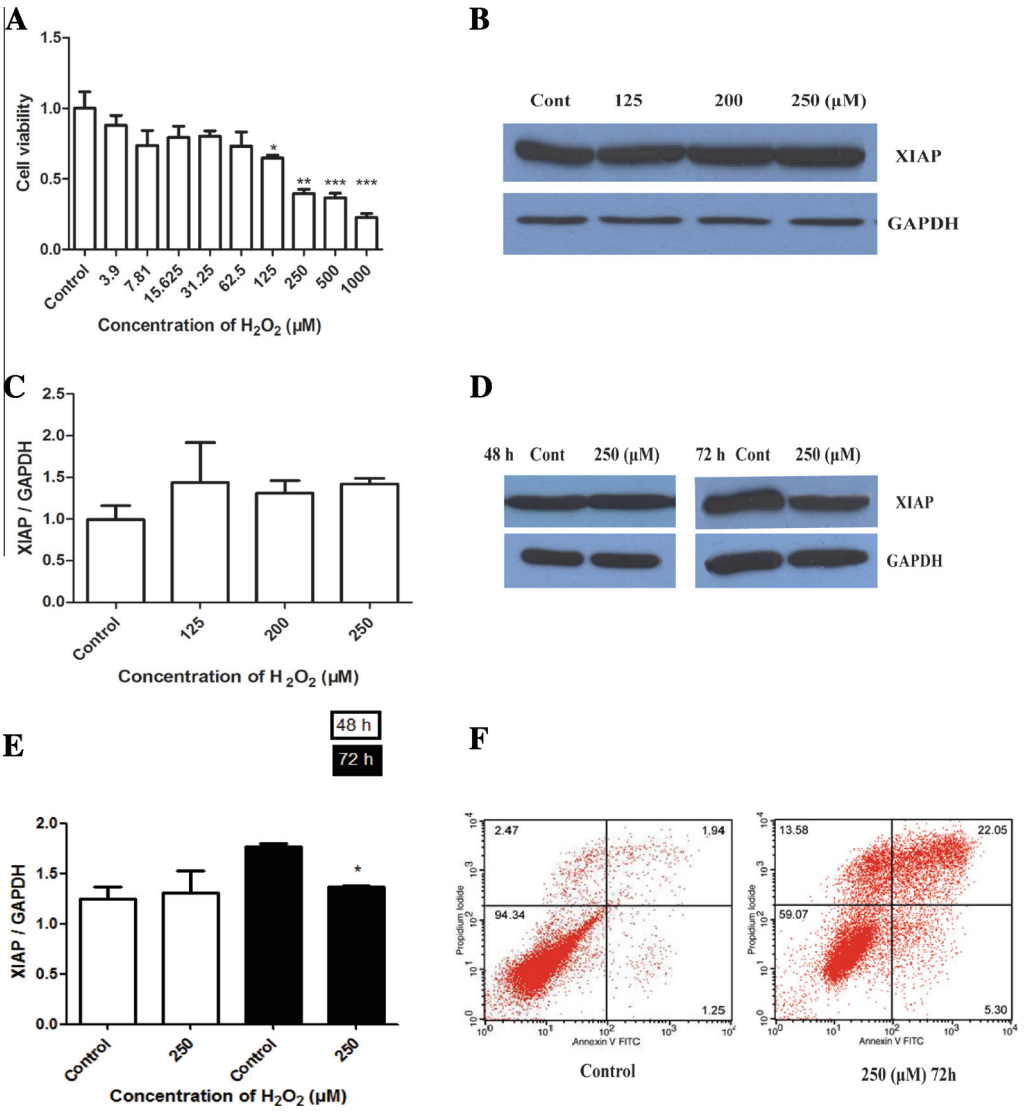

Fig. 1. Cells were treated with various concentrations of H_2O_2 and then the cell viability was assessed by an MTT assay (A). Representative western blots of XIAP for 24 h (B), 48 h and 72 h (D). Quantification was performed after 24 h (C), 48 h and 72 h (E) of H_2O_2 exposure to measure XIAP levels (F). Annexin V-FITC/PI assay shows cell apoptosis by flow cytometry. Exposure of cells to 250 μM H_2O_2 for 72 h increased cell apoptosis to 22.05% while the control was 1.94%. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

determine the percentage of cells within a population, which is actively undergoing programmed cell death. These cells lose their membrane asymmetry during early apoptosis. Briefly, 1×10^6 cells were analyzed after 72 h exposure, including control cell cultures. The cells were labeled with annexin V-FITC/PI. The annexin V-FITC−/PI− population was regarded as normal healthy cells, while annexin V-FITC+/PI− indicated early apoptosis, annexin V-FITC+/PI+ indicated late apoptosis, and annexin V-FITC−/PI+ indicated necrosis. Approximately 1×10^4 events were acquired for each experimental time point by a FACScan flow cytometry (Becton Dickinson, Heidelberg, Germany).

2.3. Western blotting

Samples were separated by 7.5% or 10% SDS–polyacrylamide gel electrophoresis and then transferred onto Hybond-C Extra nitrocellulose membranes (Amersham Biosciences, USA). Blots were blocked at room temperature for 1 h with 10% dry milk and 0.1% bovine serum albumin (BSA; Fraction V) in PBS, and then incubated at 4 °C overnight with primary antibodies diluted in PBS containing 0.1% BSA. After washing, blots were incubated with a horseradish peroxidase-conjugated secondary antibody diluted in blocking buffer and developed using ECLTM Western Blotting Detection Reagents (Amersham Biosciences, USA). Semi-quantitative analysis of immunoblots was performed by densitometry using ImageJ (National Institutes of Health, Bethesda, MD, USA). Protein levels were normalized to the loading control. Each experiment was performed in triplicate.

2.4. Immunocytochemistry

Transfected and H_2O_2 treatments of un-transfected PDLCs were fixed with 4% paraformaldehyde for 15 min on ice and then permeabilized with 0.1% Triton X-100 for 30 min at room temperature. Cells transfected with empty GFP vector were labeled using a polyclonal rabbit primary antibody to GFP (Abcam, UK). Anti-rabbit

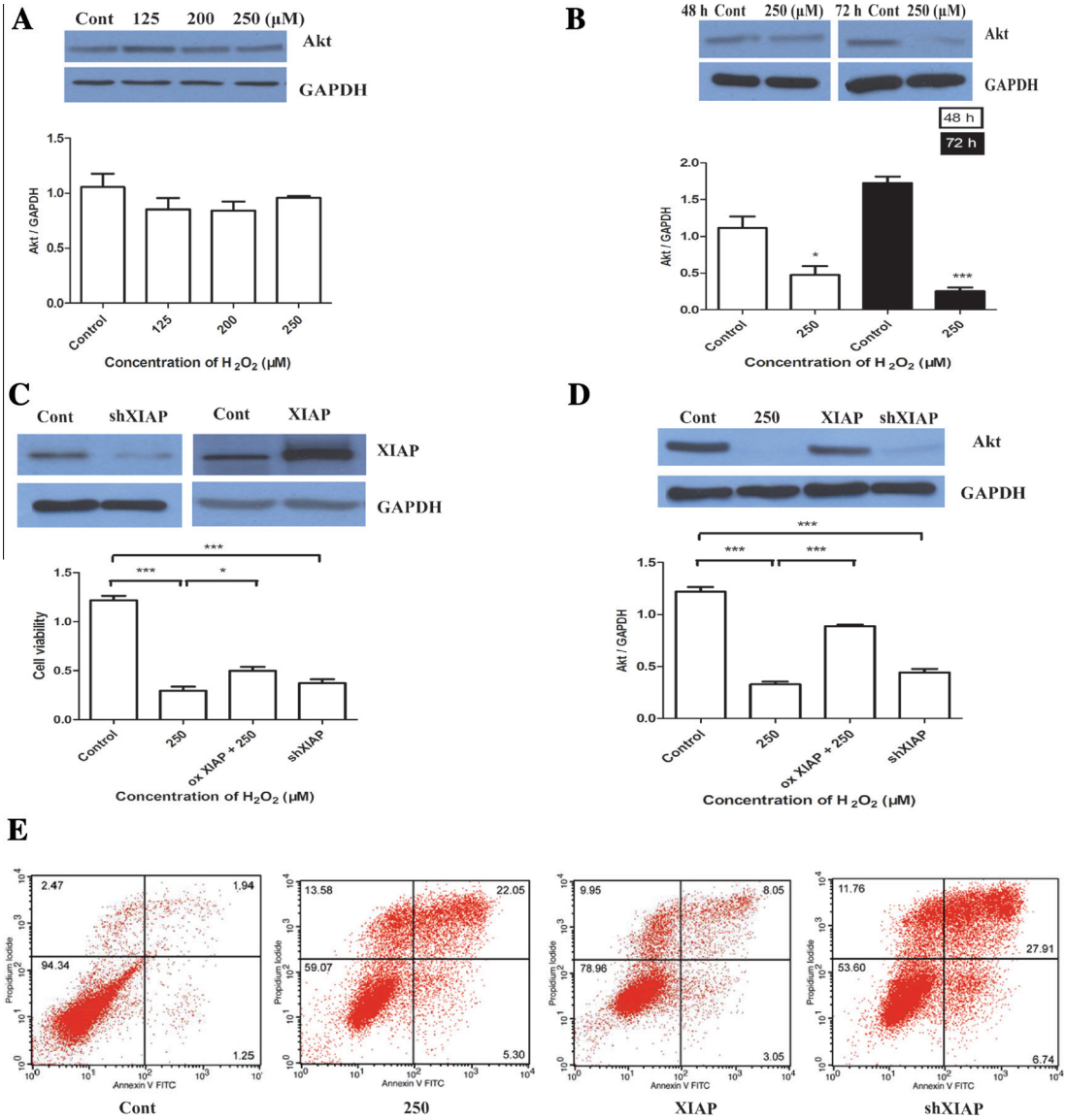

Fig. 2. Representative western blots of Akt for 24 h (A), 48 h and 72 h (B). Quantification was performed after 24 h (B), 48 h and 72 h (C) of H_2O_2 exposure to measure Akt levels. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. XIAP levels were measured by western blotting after knock-down (shXIAP) or overexpression (XIAP) of XIAP, Cont indicates untransfected PDLCs (C). Untransfected and transfected cells were treated with 250 μM H_2O_2 for 72 h for assessment of cell viabilities (C). Overexpression of XIAP partially rescued the reduction of AKT while downregulation of XIAP induced a decrease of AKT (D). Apoptosis ratio was detected for untransfected cells (Control), untransfected cells with 72 h of 250 μM H_2O_2 treatment (250), overexpression of XIAP with 72 h of 250 μM H_2O_2 treatment (XIAP), silence of XIAP without any treatments (shXIAP).

Alexa 488-, anti-mouse Alexa 568-conjugated secondary antibodies (Invitrogen, USA) and 5 μg/μL of Hoechst 33258 (Thermo, USA) were used to visualize GFP, AKT and pAKT, cell nuclei, respectively. All primary and subsequently secondary antibodies or Hoechst 33258 were incubated with cells at 37 °C for 1 h spaced with multiple PBS washes. For image acquisition, coverslips were mounted on glass slides in Fluoromount-G (Southern Biotech, USA) and were imaged with a 42× objective Nikon Eclipse 90i microscope (USA).

2.5. Transfection

PDLCs were seeded at 5×10^6 cells/10-cm culture dish and grown for 1 day in α-MEM prior to transfection with Lipofectamine 2000 (Invitrogen) or jetPEI (Polyplus). Plasmid DNA was used to transfect the cells, including 5 μg pcDNA3 carrying XIAP (for overexpression of XIAP) or 5 μg pLVTHM carrying short hairpin RNA against XIAP (shXIAP). The transfection efficiency was checked by expression of the green fluorescent protein reporter observed by fluorescence microscopy. Cells were used in experiments at 44–50 h after transfection.

2.6. Statistical analysis

Unless specified otherwise, all data were expressed as the mean ± standard deviation from at least three independent experiments. Both MTT assay and western blot data were analyzed using Graphpad 5.0 software. One-way analysis of variance was used to verify the equality of several measures when the number of quantitative variables compared was greater than two, verifying in each case whether the variances were homogeneous. Statistical significance were accepted at $p < 0.05$.

3. Results

3.1. Effects of H_2O_2 on PDLC viability

The MTT assay demonstrated a significant concentration-dependent decrease of viability following H_2O_2 exposure at 3.9, 7.81, 15.625, 31.25, 62.5, 125, 250, 500, and 1000 μM for 24 h (Fig. 1A). Compared with the control group, there was a significant reduction of cell viability in the 125-μM group ($p < 0.05$).

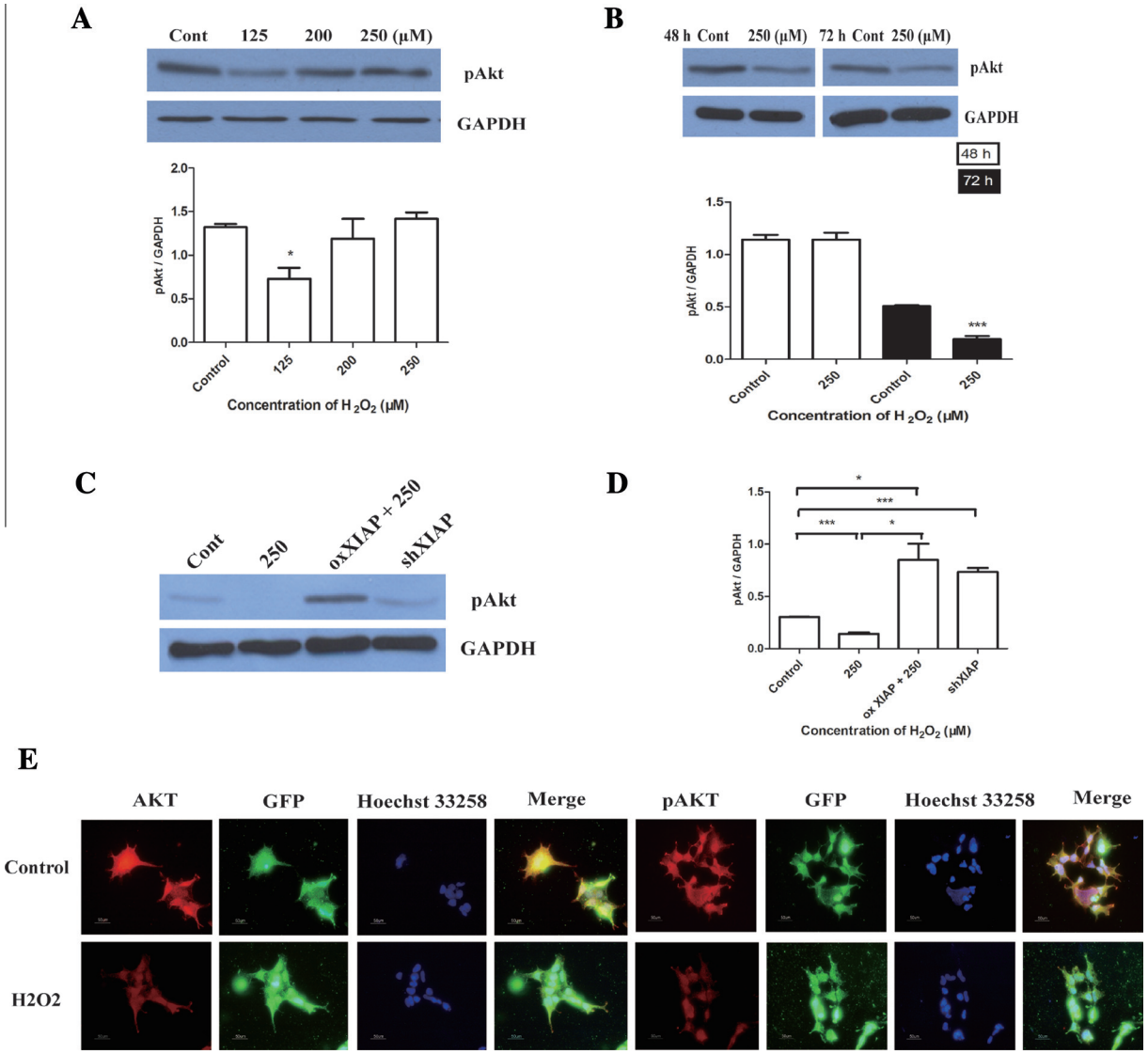

Fig. 3. Representative western blots and quantification of pAkt for 24 h (A), 48 h and 72 h (B). Overexpression of XIAP partially rescued the reduction of pAKT while downregulation of XIAP induced increases of pAKT (D), and representative immunocytochemistry of 250 μM H_2O_2 for 72 h induced a decrease of AKT and pAKT levels (E). Scale bar represents 50 μM. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Additionally, as the concentration of H_2O_2 increased to 250 μM, so did the reduction of cell viability ($p < 0.01$). As the concentration of H_2O_2 increased to 500 and 1000 μM, there was a concentration-dependent decrease of cell viability ($p < 0.001$). Apoptosis ratio was detected by PI/annexin V-FITC via flow cytometry, as showed in Fig. 1F, the apoptosis ratios were 1.94%, 22.05% for control cells with 72 h of 250 μM H_2O_2 treatment, respectively.

3.2. Effects of H_2O_2 on XIAP, AKT, pAKT, and JNK2 levels

After 24 h of treatment with 125, 200, and 250 μM H_2O_2, compared with the control group, expression of XIAP, AKT, and JNK2 showed no detectable changes ($p > 0.05$) as shown in Figs. 1C, 2B, and 4A, respectively. However, 24 h of exposure to H_2O_2 at a concentration of 125 μM, but not 200 or 250 μM, induced a significant decrease of pAKT protein levels ($p < 0.05$; Fig. 3A). After treatment with 250 μM H_2O_2 for 72 h but not 48 h, there was a significant decrease of XIAP protein levels ($p < 0.05$; Fig. 1E). After 48 h of exposure, there was also a significant decrease of both AKT and JNK2 protein levels ($p < 0.05$) as shown in Figs. 2B and 4B, respectively. Additionally, after 72 h of exposure, both Akt and JNK2 protein levels decreased significantly as shown in Figs. 2B and 4B, respectively.

3.3. Effects of overexpression and knockdown of XIAP protein on Akt, pAkt, GSK3β and JNK2 levels

XIAP protein upregulations and downregulations were confirmed by western blotting (Fig. 2C), Knock down of XIAP expression by shXIAP induced a remarkable reduction of PDLC viability ($p < 0.001$; Fig. 3C). Conversely, overexpression of XIAP partially rescued the decrease of cell viability ($p < 0.05$; Fig. 3C) that was induced by 72 h of 250 μM H_2O_2 treatment. Meanwhile, apoptosis ratio was detected by PI/annexin V-FITC via flow cytometry, as showed in Fig. 2E, the apoptosis ratios were 1.94%, 22.05%, 8.05% and 27.91% for control cells, 72 h of 250 μM H_2O_2 treatment for

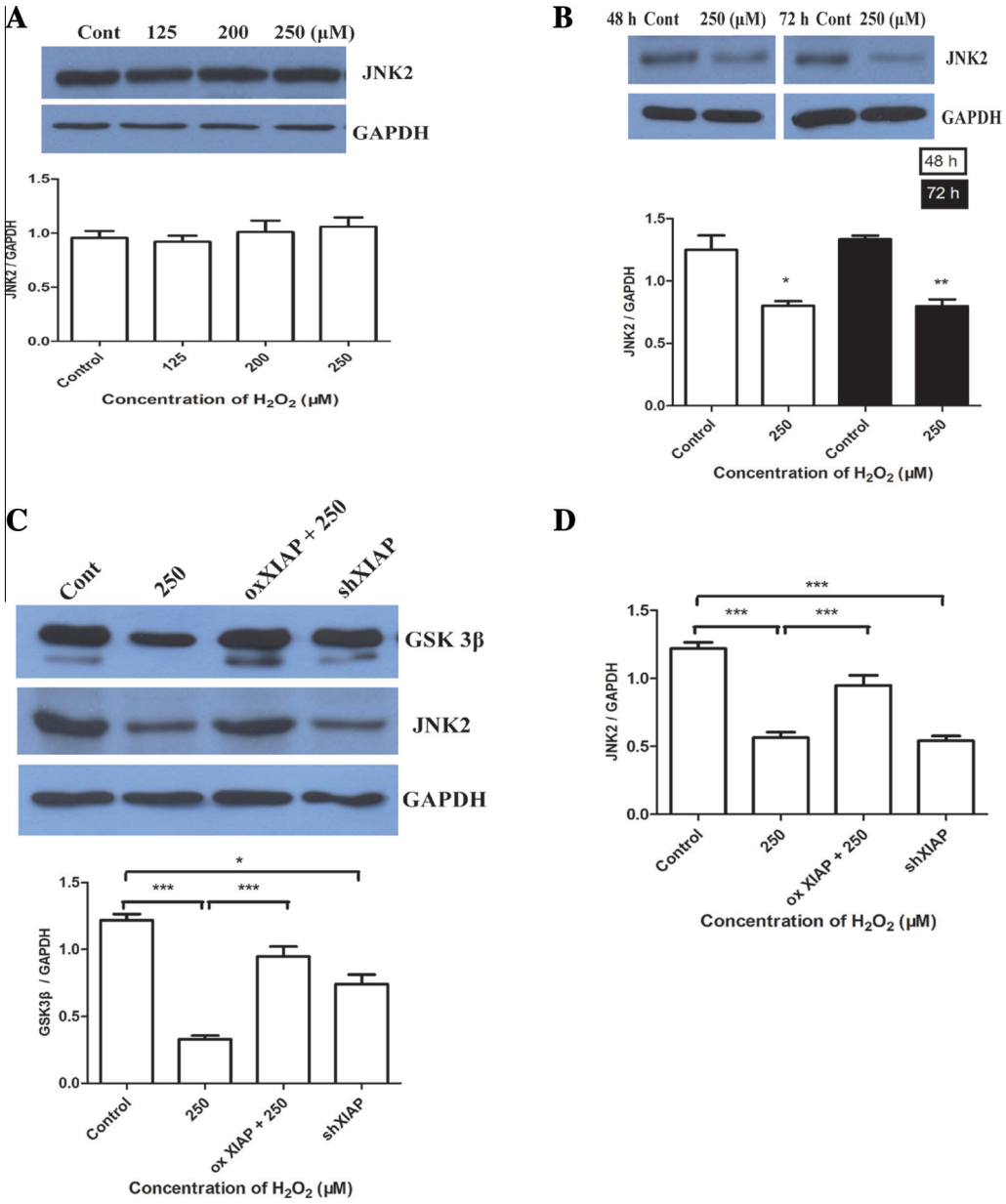

Fig. 4. Representative western blots and quantification of JNK2 for 24 h (A), 48 h and 72 h (B). Overexpression of XIAP partially rescued the reduction of GSK3β (C) and JNK2 (D) while downregulation of XIAP induced a decrease of GSK3β(C) and JNK2 (D). $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

normal cells, 72 h of 250 μM H_2O_2 treatment for overexpression of XIAP cells, and silence of XIAP cells, respectively.

After 72 h of H_2O_2 exposure, downregulation effects were observed for XIAP ($p < 0.05$; Fig. 1E), AKT ($p < 0.001$; Fig. 2D), pAKT ($p < 0.001$; Fig. 3D), GSK3β ($p < 0.001$; Fig. 4C), and JNK2 ($p < 0.001$; Fig. 4D), additionally, the down regulation effects of 72 h of H_2O_2 exposure for AKT and pAKT were confirmed by immunocytochemistry (Fig. 3). In contrast, these downregulation effects were profoundly recovered by overexpression of XIAP ($p < 0.001$). Knock down of XIAP also downregulated the expression levels of AKT, GSK3β, and JNK2 ($p < 0.001$) as shown in Figs. 3D and 4C, D, respectively. Simultaneously, XIAP knock down upregulated pAKT levels ($p < 0.001$; Fig. 3D).

4. Discussion

XIAP levels did not change after 24 h of treatment with 125, 200, and 250 μM H_2O_2. However, we observed a decrease of PDLCs viability after exposure to 125, 250, 500, and 1000 μM H_2O_2 for 24 h. Nonetheless, we checked the cellular morphology for 24 h, 48 h and 72 h of H_2O_2 treatments with the concentrations of 125, 200, and 250 μM, respectively, microscopy, showing that, PDLCs apoptotic bodies were observed obviously only in the 72 h H_2O_2 treatment group compared with control group (data not shown). The 72 h 250 μM H_2O_2 induced apoptosis ratio was checked by flowcytometry, Interestingly, we observed a greater modulation of pAKT at lower concentration although viability was only impacted at concentrations greater than 250 μM. Such decrease of pAKT might be regarded as due to the oxidative stress induced by H_2O_2 at low concentrations.

Phosphoinositide-dependent kinases have been shown to be responsible for threonine-308 phosphorylation of AKT [16]. However, this phosphorylation alone is not sufficient for full AKT activation that requires PtdIns-3, 4, 5-P3-dependent serine-473 phosphorylation. In terms of XIAP overexpression partially recovering the reduction of JNK2 and GSK3β levels, we assume that XIAP might be involved in controlling JNK and GSK3 pathways. Furthermore, it is interesting to consider whether PtdIns-3, 4, 5-P3 is required for activation of JNK and GSK3 pathways.

Previously, it was demonstrated [9,12] that XIAP was a physiological substrate of Akt. Akt interacted with and phosphorylated XIAP at serine 87. Phosphorylation of XIAP by Akt inhibited both its autoubiquitination and cisplatin-induced ubiquitination. These effects reduced XIAP degradation and the increased levels of XIAP are associated with decreased cisplatin-stimulated caspase-3 activity and programmed cell death.

In the present study, overexpression of XIAP prevented H_2O_2 induced levels of AKT and pAKT levels from decreasing. Previous work demonstrated that overexpression of XIAP had been shown to protect Chinese hamster ovary and RAT-1 cells against menadione or growth factor withdrawal-mediated apoptosis [17]. XIAP overexpression also suppresses apoptosis in HeLa cells induced by transient transfection with interleukin 1b-converting enzyme [18] and apoptosis induced by the Sindbis virus [19].

In the present study, overexpression of XIAP not only rescued the decrease of AKT and pAKT protein levels induced by H_2O_2, but also the cell viabilities, and the apoptosis ratio. Nevertheless, we found that knockdown of XIAP induced a decrease of AKT levels while increasing the pAKT level.

Recently, it has been shown that TGF-β signaling pathway can promote apoptosis through the modulation of the PI3-K/Akt pathway by down-regulating the XIAP [20,21]. AKT is a serine/threonine protein kinase that plays a central role in the PI3-K/AKT survival pathway. It is also possible that AKT regulates XIAP via NF-κB in endometrial cells. AKT activity can induce NF-κB mobilization and activation by IκB phosphorylation [22–24], NF-κB plays a significant role in immune regulation and may participate to the mechanism by which the fetus avoids maternal rejection throughout pregnancy.

Reciprocally, phosphorylation of XIAP by AKT has been shown to protect XIAP from ubiquitination and degradation in cancer cells [25]. Recent studies suggested that there is an intricate, coordinated regulatory system at play between XIAP and the AKT signaling pathway through a feedback mechanism.

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