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# Protective Effect of Propofol against Hypoxia-reoxygenation Injury in HaCaT Human Keratinocytes

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**The aim of this study was to determine the beneficial effect of propofol on human keratinocytes that have undergone hypoxia reoxygenation (H/R) injury and to investigate whether autophagy is associated with the protective mechanism. Thus, we evaluated how propofol influences the intracellular autophagy and apoptosis during the H/R process in the HaCaT cells. The cultured human keratinocyte cells were exposed to 24 h of hypoxia (5% $CO_2$, 1% $O_2$, 94% $N_2$) followed by 12 h of reoxygenation (5% $CO_2$, 21% $O_2$, 74% $N_2$). The experiment was divided into 4 groups: (1) Control=Normoxia ; (2) H/R=Hypoxia Reoxygenation ; (3) PPC+H/R=Propofol Preconditioning+Hypoxia Reoxygenation; (4) 3-MA+PPC+H/R=3-MA-Methyladenine+Propofol Preconditioning+Hypoxia Reoxygenation. In addition, Western blot analysis was performed to identify the expression of apoptotic pathway parameters, including Bcl-2, Bax, and caspase 3 involved in mitochondrial-dependent pathway. Autophagy was determined by fluorescence microscopy, MDC staining, AO staining, and western blot. The H/R produced dramatic injuries in keratinocyte cells. In our study, the viability of Propofol in H/R induced HaCaT cells was first studied by MTT assay. The treatment with 25, 50, and 100 μM Propofol in H/R induced HaCaT cells enhanced cell viability in a dose-dependent manner and 100 μM was the most effective dose. The Atg5, Becline-1, LC3-II, and p62 were elevated in PPC group cells, but H/R-induced group showed significant reduction in HaCaT cells. The Atg5 were increased when autophagy was induced by Propofol, and they were decreased when autophagy was suppressed by 3-MA. These data provided evidence that propofol preconditioning induced autophagy and reduced apoptotic cell death in an H/R model of HaCaT cells, which was in agreement with autophagy playing a very important role in cell protection.**

**Key words: propofol, hypoxia-reoxygenation, keratinocytes, apoptosis, autophagy**

## Introduction

Tissue hypoxia-reoxygenation (H/R) damage is a feature common to many skin diseases including pressure ulcers and cancer. It also occurs as an outcome of surgery and hemodynamic shock. Ischemia-reperfusion injury initiates stress pathways that ultimately result in programmed cell death [1]. The underlying mechanisms of hypoxia reoxygenation injury are multifaceted, but recent studies point towards a central role of reactive oxygen species,

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which are produced during the hypoxia reoxygenation and may damage cells by promoting DNA damage, lipid peroxidation, and oxidation of proteins [2,3]. Hypoxia during ischemia and subsequent reoxygenation upon reperfusion are thought to be the major culprits contributing to reactive oxygen species production, which leads to uncontrolled oxidative stress and subsequent cell apoptosis [4,5].

Propofol, a widely used intravenous anesthetic drug, has been demonstrated to have protective properties in several in vitro [6-9] and in vivo [10-12] models of various types of hypoxia-reoxygenation injury. Propofol is also used as a sedative for ICU patients. Propofol has a structural similarity to the endogenous antioxidant vitamin E and exhibits antioxidant activities [13]. Thus, approaches to lessen hypoxia reoxygenation injury have been extensively studied, and administration of pharmacological agents such as anesthetics was demonstrated to produce pharmacologic preconditioning against hypoxia reoxygenation injury [14,15].

Autophagy is an intracellular, evolutionarily conserved catabolic process for degrading and recycling long-lived proteins and damaged organelles [16]. Autophagy activity remains at a low level under physiological conditions and can be activated in response to stress conditions such as starvation, oxidative stress, and hypoxia-reoxygenation [17-19]. It is also associated with the pathological mechanisms involved in many diseases. The presence of autophagosomes in dying cells has implicated autophagy in the cell death process. In response to cellular stress, autophagy is up-regulated and can provide an adaptive strategy for cell survival, but may also directly or indirectly lead to cell demise [20,21]. While keratinocyte apoptosis and necrosis have been shown to occur during hypoxia, the role of keratinocyte autophagy remains controversial. In addition, the details of the apoptosis and autophagy mechanisms underlying the effects of propofol on H/R-induced keratinocytes remain unknown. In the present study, we investigated how propofol influences the intracellular autophagy and apoptotic cell death during the H/R process in the HaCaT cells.

## Materials and Methods

### Reagents

Propofol (2, 6-diisopropylphenol) diluted with dimethyl sulfoxide (DMSO), Hoechst 33342 was purchased from Sigma. The following reagents were obtained commercially: 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl tetrazolium bromide (MTT), acridine orange, ,monodansylcadaverine (MDC), 3-methyladenine (3-MA, class III PI3K inhibitor) was obtained from Calbiochem (La Jolla, CA, USA). Annexin Vfluorescein isothiocyanate (FITC) apoptosis detection kit was obtained from Biovision (Milpitas, CA, USA). Antibodies used in the study were as follows: Cleaved Caspase 3 (1:1,000), : Cleaved Caspase 9 (1:1,000), : Bcl-xl (1:1,000), : Bax (1:1,000), LC3 (1:3,000), : Becline-1 (1:1,000) from Abcam, p62 (1:1,000), Atg5 (1:500), from Santa Cruz. Secondary antibodies against rabbit (1:3,000), and mouse (1:3,000), immunoglobulins were purchased from Bio-Rad.

### Cell culture

Human keratinocytes (HaCaT) were obtained from the American Type Culture Collection (ATCC, Manassas, USA). Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 10% inactivated fetal bovine serum (FBS, GIBCO) containing 500 µg/ml penicillin and 500 µg/ml streptomycin (GIBCO), and cells were incubated at 37°C in a humidified atmosphere with 5% $CO_2$. Media were changed every 3 days.

### Treatment of propofol

Propofol which were made by dissolving them in DMSO were kept frozen at -4°C until use. The stock was diluted to their concentration with DMEM when needed. Prior to propofol treatment cells were grown to about 80% confluence and then exposed to propofol at different concentrations (0, 25, 50, 100 M) for 24 h. Cells grown

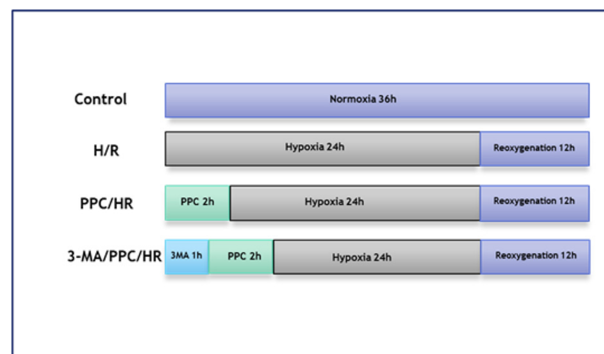


**Fig. 1.** The experiment protocols. In vitro experiments are shown. Control = Normoxia ; H/R = hypoxia/Reoxygenation; PPC = Propofol Preconditioning; 3-MA = 3-Methyladenine.

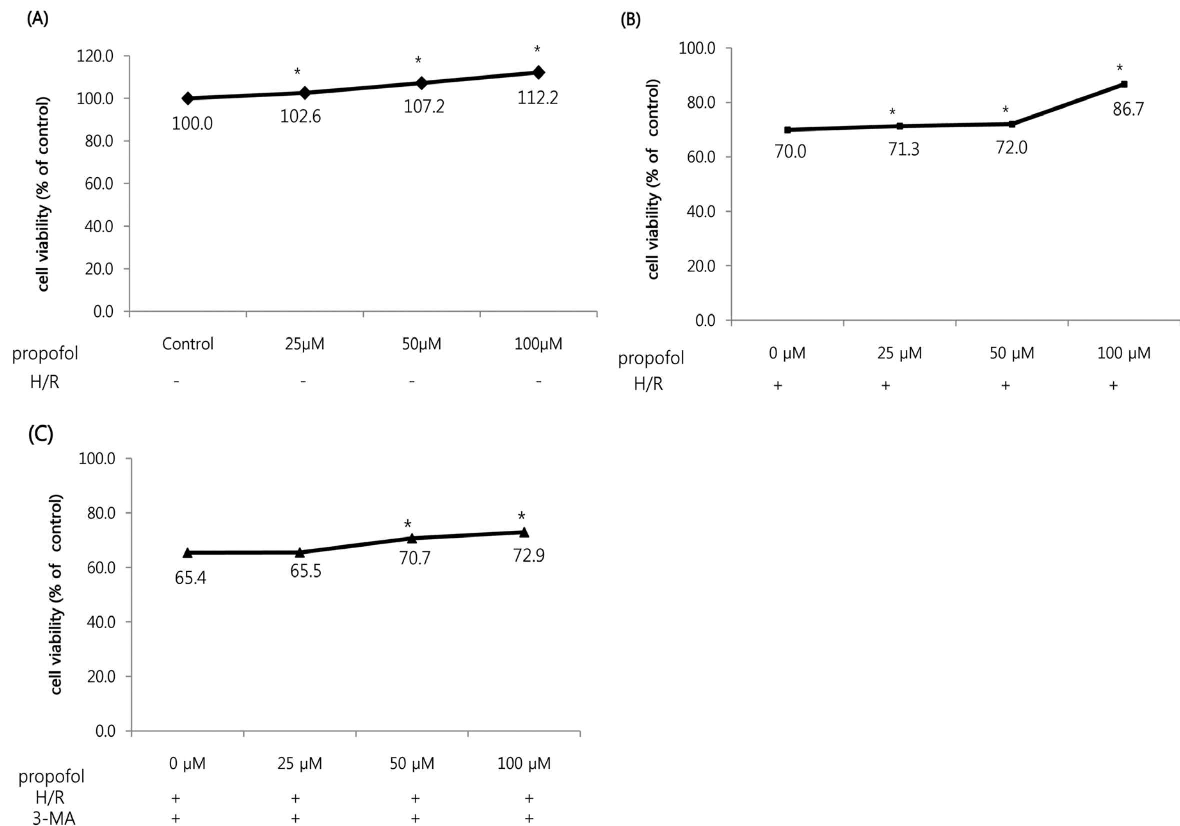


**Fig. 2.** (A) Effect of propofol preconditioning on cell viability. The normal keratinocytes were treated with different concentrations (0, 25, 50, 100 μM) of propofol for 36 h. *, $p<0.05$ (B) Effect of hypoxia-reoxygenation injury and propofol on cell viability. The cells were pre-treated with increasing concentrations of propofol (0, 25, 50, 100 μM) after 24 h of hypoxia and 12 h of reoxygenation. *, $p<0.05$ (C) HaCaT cells were pre-treated 3-MA with various doses of propofol and exposed to H/R. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenylterazolium bromide (MTT) assay. *, $p<0.05$

in medium containing an equivalent amount of DMSO without propofol served as control. The groups were randomly divided into the following groups: Control: cells were incubated in normoxia (5% $CO_2$, 21% $O_2$, and 74% $N_2$) without propofol treatment. H/R: cells were exposed to 24 h of hypoxia (5% $CO_2$, 1% $O_2$, and 94% $N_2$) followed by 12 h of reoxygenation (5% $CO_2$, 21% $O_2$, and 74% $N_2$). PPC/HR: cells pretreated with propofol were exposed to 24 h of hypoxia followed by 12 h of reoxygenation. 3-MA/PPC/HR: cells pretreated with 3-MA and propofol were exposed to 24 h of hypoxia followed by 12 h of reoxygenation (Fig. 2B).

## MTT assay

Cell viability assay was measured using a quantitative colorimetric assay with thiazolyl blue tetrazolium bromide (MTT, amresco), showing the mitochondrial activity of living cells. HaCaT cells ($3x10^4$) were seed in 96-well plates. After drug treatment as indicated, cells were incubated with 300 μl MTT (final concentration 0.5 mg/ml) for 1.5 h at 37°C. The reaction was terminated by addition of 200 μl DMSO. Cell viability was measured by an ELISA reader (Tecan, Mnnedorf, Switzerland) at 570 nm excitatory emission wavelength.

## Flow cytometer analysis

Quantification of apoptotic cells was determined by Annexin V-FITC/PI staining. The adherent cells were collected by centrifugation and then resuspended in 500 μl 1x binding buffer. Then they were stained with 5 μl Annexin VFITC and 5 μl propidium iodide (PI) (50 μ g/ml) and incubated at room temperature for 5 min in the dark. The cells were analyzed by a flow cytometry (Becton Dickinson. FACS Vantage SE, Sanjose, USA). The results were shown as quadrant dot plots with intact cells (Annextin V-/PI-), early apoptotic cells (Annextin V+/PI-),

late apoptotic cells (Annextin V+/PI+) and necrotic cells (Annextin V-/PI+). The number of each kind of cells was expressed as percentages of the number of total stained cells.

### Fluorescence microscopy

Cells were grown on coverslips and treated with propofol. After 24 h, cells were stained with 0.05 mM MDC, a selective fluorescent marker for autophagic vacuoles, at 37°C for 1 h. The cellular fluorescence changes were observed using a fluorescence microscope (Axioskop, Carl Zeiss, Germany). For further detection of the acidic cellular compartment, we used acridine orange, which emits bright red fluorescence in acidic vesicles but fluoresces green in the cytoplasm and nucleus. Cells were stained with 1 µg/ml acridine orange for 15 min and washed with PBS. AVOs formation was obtained under a confocal microscope LSM 700 (Carl Zeiss, Germany).

### Western blot analysis

Cells (2 x $10^6$) were washed twice in ice-cold PBS, resuspended in 200 µl ice-cold solubilizing buffer [300 mM NaCl, 50 mM Tris-Cl (pH 7.6), 0.5% Triton X-100, 2 mM PMSF, 2 µl/ml aprotinin and 2 µl/ml leupeptin] and incubated at 4°C for 30 min. The lysates were centrifuged at 14,000 revolutions per min for 15 min at 4°C. Protein concentrations of cell lysates were determined with Bradford protein assay (Bio-Rad, Richmond, CA, USA) and 20 µg of proteins were resoved by 10% SDS/PAGE. The gels were transferred to Polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) and reacted with appropriate primary antibodies. Immunostaining with secondary antibodies was detected using SuperSignal West Femto (Pierce, Rockford, IL, USA) enhanced chemiluminescence substrate and detected with Alpha Imager HP (Alpha Innotech, Santa Clara, USA).

### Statistical analysis

Three independent experiments were performed for each experimental group and each experiment was performed in triplicate. The results of the experimental and control groups were cpmpared for statistical significance ($p<0.01$ and 0.05) using paired T-test statistical method by SPSS for Win 12.0 for summary data.

## Results

### Propofol improved the cell viability of H/R–induced HaCaT cells

The effect of propofol on HaCaT cells was investigated over a wide concentration range. In particular, the H/R-dependent programed cell death was suppressed by propofol, a potent antioxidant. The viability of propofol treated HaCaT cells increased in a dose-dependent manner. Propofol did not show any significant toxic effect on the HaCaT cells (Fig. 2A). We next investigated the effect of various concentrations of propofol (0, 25, 50, and 100 µ M) on the H/R-induced cells and discovered that propofol significantly protected the HaCaT cells from H/R-induced cell cytotoxicity. However, propofol significantly enhanced cytotoxicity with at doses of 200 and 400 µM. We pretreated HaCaT cells with various doses of propofol (below 200 µM) and exposed the cells to H/R and then we measured cell viability by the MTT assay. As shown in (Fig. 2B), cell proliferation was markedly inhibited after H/R treatment compared to the control ($P<0.05$), and was significantly improved by propofol. Propofol at 100 M gave the optimal effect, improving cell viability by approximately 70% ($P<0.05$). All subsequent experiments were performed with 100 µM Propofol. The role of autophagy in the H/R damage to HaCaT cells was further confirmed by the autophagy inhibitor 3-MA, an inhibitor of class III phosphoinositide 3-kinase (PI3K). Pretreatment with 3-MA (5 mM) 1 h before H/R (Reoxygenation 12 h after hypoxia for 24 h) significantly reduced H/R-induced in HaCaT cells. A decrease in cell viability was accompanied by an increase in cell death, indicating that the inhibition of autophagy by 3-MA sensitized HaCaT cells to H/R damage (Fig. 2C).

### Propofol protected against H/R–induced apoptosis in HaCaT cells

The effect of propofol on apoptosis was examined by Hoechst33342 staining of HaCaT cells exposed to normoxia (control), PPC (propofol preconditioning), H/R (hypoxia reoxygenation) and 3-MA/PPC/H/R combined with propofol after a 3-MA pre-treatment. Cells were viewed under a fluorescence microscope (x400) (Fig. 3A). The majority of HaCaT cells in the control and PPC groups

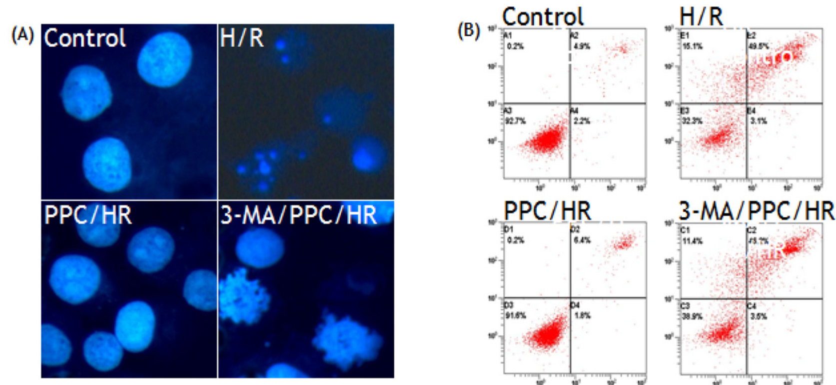


**Fig. 3.** (A) Morphological change of in H/R-induced HaCaT cells preconditioned with propofol (100 µM) and 3-MA. H/R-induced HaCaT cells preconditioned with propofol and 3-MA were observed by fluorescence microscopy. Apoptotic bodies in the propofol preconditioning group were markedly reduced. (B) Detection of apoptosis and necrosis with Annexin-V-FITC and propidium iodide staining. Every group of cells with Annexin-V and propidium iodide staining were measured by flow cytometry. Control = Normoxia; H/R = Hypoxia/ Reoxygenation; PPC = propofol preconditioning; 3-MA = 3-Methyladenine.

showed normal morphology with round regular nuclei. In contrast, apoptotic bodies were seen in the H/R group and the 3-MA/PPC/HR group cells. However, the pretreatment with propofol effectively reduced HaCaT cell apoptosis according to the restored morphology. Annexin-V FITC/PI staining quantitatively confirmed the anti-apoptotic effects of propofol (Fig. 3B). Compared with control group, the portion of Annexin-V(+)/PI(-) cells in H/R group increased from 3.1% to 49.5% ($P<0.05$). However, pretreatment with propofol 2 h prior to H/R significantly attenuated the percentage of Annexin-V(+)/PI(-) cells to 6.4% ($P<0.05$), demonstrating the anti-apoptotic effect of propofol.

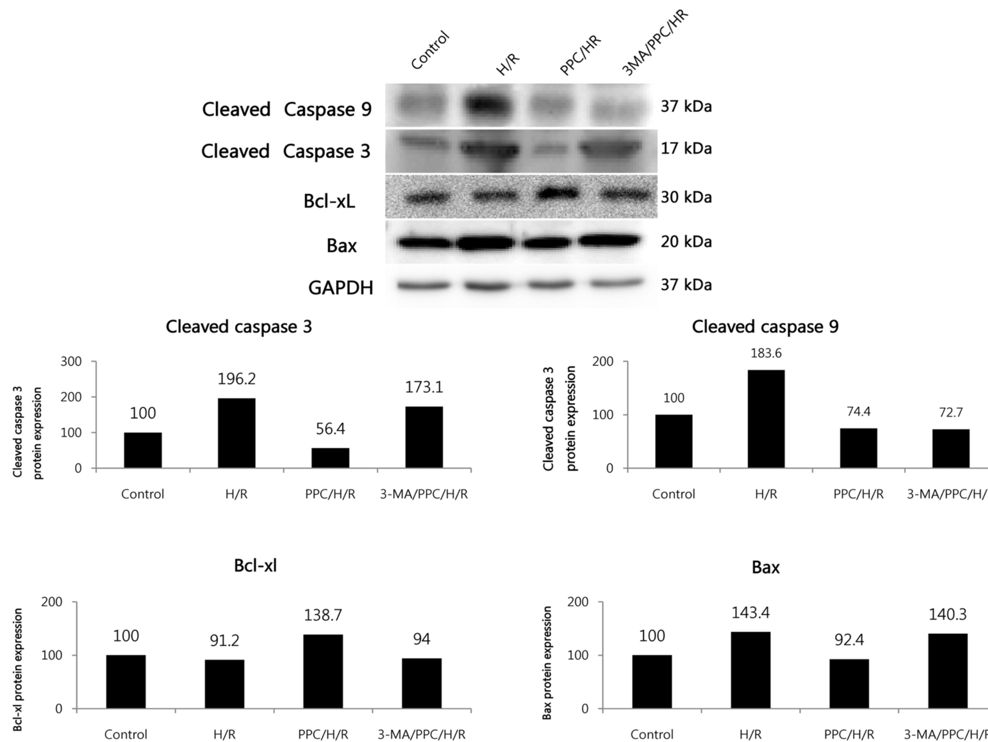


**Fig. 4.** (A)Western blot analysis. Expression of Cleaved Caspase 3, Cleaved Caspase 9, Bcl-xl and Bax in H/R-induced HaCaT cells preconditioned with propofol and 3-MA. (B) Quantification of Cleaved Caspase 3, Cleaved Caspase 9, Bcl-xl and Bax. Each band shown above was quantified by the densitometric scan. Control = Normoxia; H/R = Hypoxia/ Reoxygenation; PPC = propofol preconditioning; 3-MA = 3-Methyladenine.

## Propofol inhibited Cleaved Caspase 3, 9 activation in H/R induced HaCaT cells

The Cleaved Caspase 3, Cleaved Caspase 9 activities in HaCaT cells was examined by western blotting. Activation of Cleaved Caspase 3, Cleaved Caspase 9 is a key up-stream event involved in the initiation and execution of apoptosis. We confirmed that Cleaved caspase 3, Cleaved Caspase 9 was involved in the H/R-induced apoptosis and the effect of propofol on the apoptosis by examining the expression of Cleaved Caspase 3, Cleaved Caspase 9 by western blot analysis (Fig. 4). Compared with the PPC group, Cleaved Caspase 3, Cleaved Caspase 9 was down-regulated in the H/R induced HaCaT cells, whereas it was up-regulated in H/R induced cells. Pretreatment with propofol antagonized this effect by inhibiting the caspase-dependent pathway.

## Propofol attenuated the apoptosis through mitochondrial dependent pathway in HaCaT cells

The mitochondria-dependent pathway of apoptosis is regulated by Bcl-xl family members, such as the anti-apoptotic protein Bcl-xl and pro-apoptotic protein Bax, which are critical for downstream caspase activation. We investigated whether mitochondria were responsible for executing H/R induced apoptosis by analyzing the expression of Bcl-xl and Bax (Fig. 4). The H/R treatment up-regulated the protein level of Bax while the level of Bcl-xl was down-regulated. Propofol treatment reduced the western density and the expression of Bax decreased at the same time, indicating that propofol can attenuate apoptosis through the mitochondrial related pathway in HaCaT cells. A wound healing assay was done to determine the migratory capability of HaCaT cells. Wound healing wasmonitored at 0 and 36 h (Fig. 5). The PPC/HR group treated with propofol showed increased wound healing capability.

## Propofol treatment leads to induction of autophagy in HaCaT cells

Prominent accumulation of autophagic specific staining MDC was observed around the nuclei in the PPC group HaCaT cells (Fig. 6A). We clarified the role of propofol-

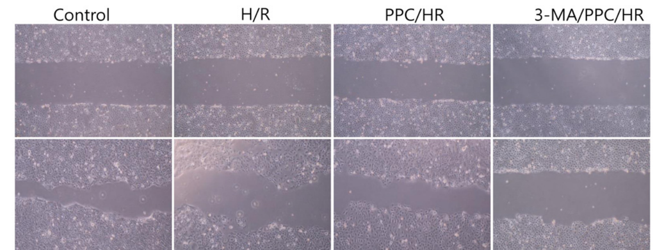


**Fig. 5.** Propofol restored cell proliferation and migration, which had been decreased by hypoxia. Investigation of cell migration capability after H/R was performed. Confluent monolayers of HaCaT cells were wounded by scratching the surface as uniformly as possible with a 1 ml pipette tip.

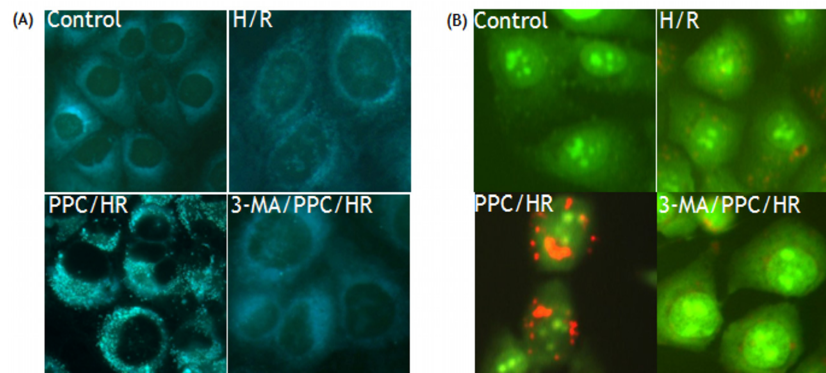


**Fig. 6.** Fluorescence microscopic (x400) analysis of autophagosome in the H/R injured HaCaT cells. H/R caused accumulation of autophagosomes containing partially digested cytoplasmic contents compared to the control group. The PPC during H/R dramatically increased formation of autophagosomes and the autophagy pathway inhibitor 3-MA blocked formation of autophagosomes by PPC. (B) Fluorescence microscopic (x400) analysis of autophagosome in the H/R injured HaCaT cells. Stained with acridine orange the green shows where the dye has stained the nucleus and the red is where the cell is starting to 'digest' parts of itself in small capsules called autophagosomes. H/R caused accumulation of autophagosomes containing partially digested cytoplasmic contents compared to the control group. The PPC during H/R dramatically increased formation of autophagosomes and the autophagy pathway inhibitor 3-MA blocked formation of autophagosomes by PPC. Control = Normoxia; H/R = Hypoxia/Reoxygenation; PPC = propofol preconditioning; 3-MA = 3-Methyladenine.

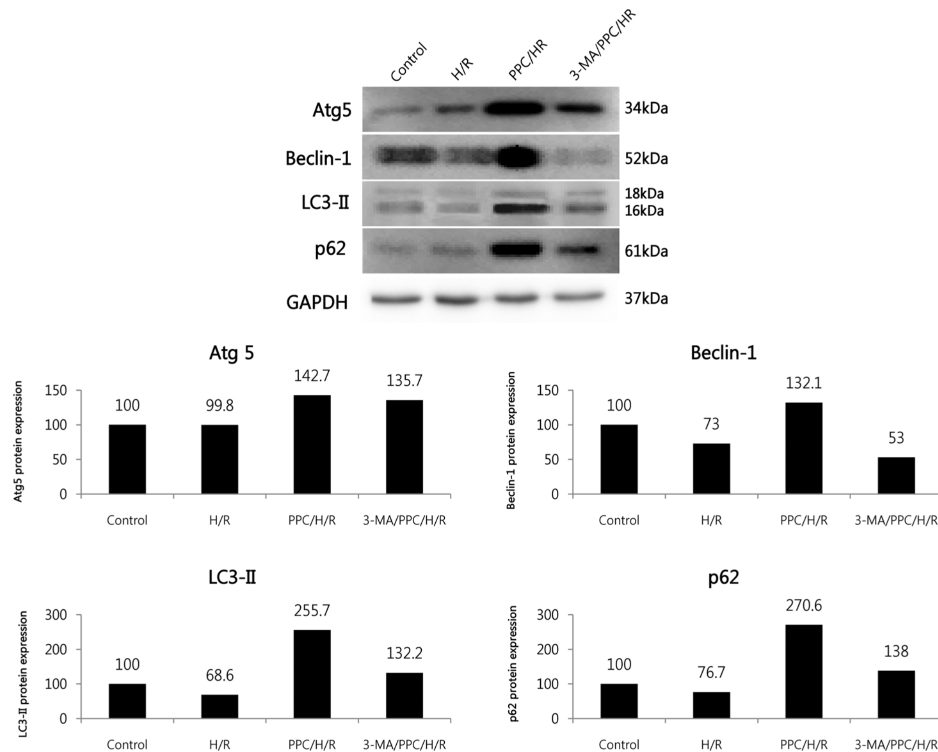


**Fig. 7.** (A)Western blot analysis. Expression of Atg5, LC3-II, Beclin-1 and p62 in H/R-induced HaCaT cells preconditioned with propofol and 3-MA. (B)Quantification of Atg5, LC3-II, Beclin-1 and p62. Each band shown above was quantified by the densitometric scan Control = Normoxia; H/R = Hypoxia/ Reoxygenation; PPC = propofol preconditioning; 3-MA = 3-Methyladenine.

induced autophagy in HaCaT cells by investigating the consequences of treatment with 3-MA, a selective autophagy inhibitor. The inhibitory effects on AVO (autophagic vacuole organelle) formation by 3-MA was confirmed by quantitatively measuring the red fluorescence ratio after AO staining. Red fluorescent spots appeared in the propofol pretreated HaCaT cells, while the control group, and the cells co-treated with 3-MA (5 mM) and the H/R group showed mainly green cytoplasmic fluorescence (Fig. 6B). We examined the activation of autophagy related protein in the H/R-induced cells by western blotting analysis. The recruitment of LC3-II to the membrane occurs via an Atg5-dependent mechanism, and thus Atg5 is essential for autophagosome formation in vivo. The levels of Atg5 and Beclin-1, LC-3 II (microtubule-associated protein 1 light chain 3 form II), and p62 were decreased in the H/R group than control group by H/R- induced apoptotic signal but the PPC group were increased (Fig. 7). The Atg5, Beclin-1, LC3-II, and p62 were increased when autophagy was induced by propofol, and they were decreased when autophagy was suppressed by 3-MA.

## Discussion

The object of the current study was to determine the beneficial effect of propofol on human keratinocytes that have undergone H/R injury and to investigate whether autophagy is associated with the protective mechanism. We showed that propofol treatment increased the proliferation of human keratinocytes with H/R injury, but the autophagy pathway inhibitor 3-MA blocked the protective effect of propofol against this injury. Previous reports have shown that the cellular protective mechanism of propofol may partly result from its ability to act as a free radical scavenger [22-24]. This study provides a new direction of research on mechanisms of propofol-mediated cytoprotection in H/R injury. Because of the antioxidative effect of propofol, preconditioning with this drug decreased the apoptotic cell death and enhanced autophagy activity in HaCaT cells under H/R injury. Pretreatment with propofol inhibited oxidative stress and subsequent apoptosis in HaCaT cells subjected to H/R injury.

Mechanisms underlying the protective effects may be largely attributed to the deactivation of mitochondrial stress pathways. Autophagy is widely known as an important process in cell physiology, for both cell survival and death. It has been reported that when organelles and portions of the cytoplasm are degraded beyond a certain threshold, autophagic cell death is induced. However, we speculated that activation of autophagy in the early period may also play a protective role by promoting survival. Oxidative stress can contribute to apoptosis which has an important role in the pathogenesis of skin H/R. Propofol pretreatment significantly inhibited the H/R induced changes. Compared with the H/R group without propofol treatment, propofol reduced the expression of apoptotic proteins towards PPC/HR group. A recent study showed that ROS play a role in the regulation of autophagy [25]. The results of the present study suggest that ROS also play a role in the activation of autophagy induced by propofol. The antioxidant propofol not only decreased H/R induced oxidative stress, but also promoted the up-regulation of autophagy.

Although many studies have focused on H/R injury in cells, an agent that could effectively protect against the cell injury remains unidentified. The functional relationship between apoptosis and autophagy is complex, in several scenarios, autophagy constitutes a stress adaptation for avoiding cell death, whereas in other cellular settings, autophagy constitutes an alternative pathway to cellular demise that is called autophagic cell death [20, 26-28]. In the ischemic phase of H/R, autophagy can be protective via AMPK activation and sequential inhibition of mTOR signaling, but reperfusion after ischemia stimulates autophagic cell death through a different pathway. However, the role of autophagy in tissue injury is still controversial. Autophagy protects cells from ischemia and reperfusion injury [29], but much ischemia and reperfusion damage is associated with autophagy-induced cell death in the heart and other organs [30]. The relationship between apoptosis and autophagy activity, elaborated above, means that all the results in our study indicate that propofol may exert a protective effect against H/R injury in HaCaT cells by directly and indirectly suppressing mitochondrial related pathway. The signal communication between the mitochondria in the protective effect of propofol is still a fascinating emerging area of investigation. The dose-dependent and time-dependent effects and mechanisms involved are still not clear. In our study, the viability of propofol in H/R induced HaCaT cells was first studied by MTT assay. The treatment of H/R induced HaCaT cells with 25, 50, and 100 μM propofol enhanced cell viability in a dose-dependent manner and 100 μM was the most effective dose. Our experiment has a number of limitations including that it is an in vitro study. Condition dependent differences may exist and the experimental H/R model of HaCaT cells is a surrogate model of I/R injury.

In conclusion, these data provided evidence that propofol preconditioning induced autophagy and reduced apoptotic cell death in a H/R model of HaCaT cells, which was in agreement with autophagy playing a very important role in cell protection. Although the current study could not establish the protection by propofol preconditioning in vivo, the data provide another model for the study of endogenous mechanisms evoked by propofol preconditioning. Finally, our results contribute further to the body of knowledge regarding the relationship between apoptosis and autophagy.

## Acknowdgements

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## Conflict of interest

The authors declare that they have no competing interest.

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