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URSODEOXYCHOLIC ACID REGULATES CASPASE-9 AND ROS PRODUCTION IN PROTECTING CARDIOMYOCYTES AGAINST HYPOXIA

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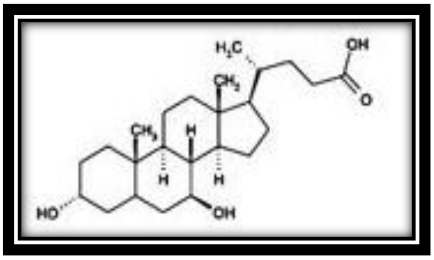
Graphical abstract



Abstract

Ursodeoxycholic acid (UDCA) is known as a therapeutic agent in treating cholestasis and liver diseases. Recently, UDCA has been suggested as a therapeutic drug for heart related diseases. Cardioprotective effect of UDCA against the development of ischemia has been studied. Yet, the mechanism of UDCA-cardioprotection is not clearly understood. Therefore, this study aimed to elucidate the mechanisms of UDCA cardioprotection against hypoxia by investigating the expression of caspase -3/-9 and ROS generation using an *in vitro* hypoxic heart model. A newborn (0-2 days old) rat heart was isolated for primary cell culture of cardiomyocytes. Hypoxia was chemically induced by using $CoCl_2$. Cardiomyocytes were then incubated with UDCA. The treated cardiomyocytes were subjected for ROS generation detection assay, QuantiGene Plex assay for caspase-3/-9 gene expression and ELISA for caspase-3/-9 protein expression. The data were analyzed by using sample paired t-test and One-way ANOVA. Our results showed that UDCA abolishes the effects on $CoCl_2$ in ROS production and UDCA downregulates *caspase*-9 protein expression in $CoCl_2$ treated cardiomyocytes. This study provides an insight of UDCA in protecting cardiomyocytes against hypoxia mediated by anti-apoptosis mechanism.

Keywords: UDCA, hypoxia, cardiomyocytes, caspase, ROS

Abstrak

Asid Ursodeoxycholic (UDCA) dikenali sebagai agen terapeutik untuk merawat kolestasis dan hati. Masa kini, UDCA dicadangkan sebagai ubat terapeutik untuk penyakit yang berkaitan jantung. Kesan UDCA terhadap iskemia telah dikaji. Walau bagaimanapun, mekanisme tindakan dalam perlindungan UDCA tidak difahami dengan jelas. Oleh itu, kajian ini bertujuan untuk menentukan mekanisme perlindungan UDCA dengan mengawal caspase-3/-9 dan generasi ROS menggunakan model hipoksia anak tikus untuk *in vitro* sel jantung. Jantung anak tikus yang baru lahir (0-2 hari) diasingkan untuk kultur sel jantung. Hipoksia telah didorong menggunakan $CoCl_2$. Sel Jantung diberi rawatan UDCA. Sel jantung yang dirawat tertakluk untuk ROS generasi eksperimen, QuantiGene Plex eksperimen untuk caspase-3/-9 gen dan ELISA untuk caspase-3/-9 protein. Data dianalisis dengan menggunakan ujian-t dan One-way ANOVA. Kajian meunjukkan UDCA menghalang kesan $CoCl_2$ ke atas pengeluaran ROS dan UDCA menurunkan ekspresi caspase-9 protein di dalam sel jantung yang telah diberi rawatan dengan

$CoCl_2$. Kajian ini memberikan gambaran mengenai mekanisme pencegahan apoptosis oleh UDCA melindungi sel jantung dari hipoksia.

Kata kunci: UDCA, hipoksia, sel jantung, caspase, ROS

1.0 INTRODUCTION

Classically bile acids are known for their role in lipid digestions and absorption in small intestine. Nowadays, bile acids are known as an important molecules that regulate glucose metabolism [1], lipids metabolism [2] and immune response [3]. In addition, bile acids have been recognized as a signaling molecules in colon cancer cells [4], hepatocytes [5] and cardiomyocytes [6]. In the small intestine, a small constituent of the hydrophilic bile acids known as ursodeoxycholic acid (UDCA) is produced. Chinese traditional practitioner encountered the importance of black bear bile as an ingredient in treating the liver and liver related diseases. The black bear bile contains high constituent of the important hydrophilic bile acid, UDCA.

UDCA is used to treat various cholestatic disorders such as interahepatic cholestasis of pregnancy, primary billary cirrhosis, cholelithiasis [7] and primary sclerosing cholangitis [8]. The use of UDCA as a drug to treat non-liver diseases such as colon carcinoma [9], alzheimer disease [10], glaucoma [11], prostate cancer [12] and osteoporosis [13] have been considered and widely studied. Miragoli et al. has demonstrated that UDCA protects cardiomyocytes against arrhythmogenic consequences of the more hydrophobic bile acid such as taurocholate [14]. Interestingly, *in vivo* studies of chronic heart failure (CHF) showed that UDCA improves patients' peripheral blood flow [15].

Hypoxia is a condition where cells experienced lack of oxygen level supply that could lead to apoptosis. Hypoxia c occurs in pathophysiology conditions, including atherosclerosis [16], ischemic disease [17] and cancer cell [18]. In human, hypoxia which occurs due to impaired blood flow would detrimentally effects organ structure and functions. This has been reported in the case of several ischemia (stroke) and myocardial ischemia (heart infarction). Furthermore, the study suggested cardiac cells are sensitive towards the changes of the oxygen supply to meet their oxidative requirements [19]. Human studies further confirmed that the failure of the oxygen circulation would result in cardiac arrest, myocardial infarction and congestive heart failure (CHF) [20].

Several mechanisms have been proposed in a higher organism as to how cells respond to hypoxia. Studies showed exposure of cells to chronic or moderate hypoxia cause an increase of Reactive Oxygen Species (ROS) generation [21]. Moreover, increase of ROS during hypoxia is known in initiating the activity of hypoxia induction factor (HIF). During hypoxia, cardiomyocytes could undergo apoptosis which is mediated by reactive oxygen species (ROS) generation [22] and caspase-9 activation [23]. Nevertheless, another study had shown that ROS exposure for a short period of time lead to ischemic adaptation in hearts of myocardial infarction animal models [24]. Heart muscle cells required constant oxygen supply; prolonged hypoxia would affect cardiac contractile protein and $[Ca^{2+}]_i$ regulation.

In hepatocytes, UDCA has been shown to have an anti-apoptotic effect and improved biochemical response in post-operational liver transplantation [25]. Meanwhile in gastric cancer cells, UDCA has been shown to activate caspase-8, caspase-3 and caspase-6 [26]. In gastrointestinal cancer cell lines (SNU601), UDCA is reported to activate ERK 1/2 and then caspases (-8,-3 and -6) and subsequently induced apoptosis [26].

Drug such as streptokinase [27], trimetazidine [28] and sulfaphenazole [29] are commonly used in treating various heart diseases. Recently, UDCA has been suggested as a candidate drug to treat cardiac diseases. However the exact mechanism of UDCA cardioprotection is not fully understood and discovered. Therefore in here we aimed to investigated anti-apoptosis mechanism of UDCA in protecting cardiomyocytes against hypoxia. We hypothesized that UDCA acts as a cardioprotective drug by regulating caspase-3, caspase-9 and ROS production of the hypoxic cardiomyocytes model.

2.0 METHODOLOGY

The primary cell culture of cardiomyocyte was isolated from 0 to 2 days old newborn Sprague-Dawley rat according to the ethics committee approval UiTM-CARE (99/2015). The cardiomyocytes were subjected for the treatment of UDCA and followed by cobalt chloride (pre-UDCA) or *vice versa*; cobalt chloride treated on cardiomyocytes first then followed by the treatment of UDCA (post-UDCA) for ROS detection. However, in subsequent experiment only pre-UDCA treatment was done. This decision was made based on the result of ROS production.

For ROS detection, 2',7'-dichlorofluorescin diacetate (DCF-DA) [30] was diluted with 5 $mmol^{-1}$ ethanol as stock solution and the working solution of the 5 μmol^{-1} DCF-DA was prepared prior to experiment. In here, ROS was detected using

inverted fluorescence microscope (Fluorescence Microscopy, Olympus IX81) and Microplate reader (Victor X5, Perkin Elmer). The cardiomyocytes were grown in 96-well plate with density of 20 000 cardiomyocytes per well (for microplate experiment) and in 6-well plate with density of 350 000 cardiomyocytes per well (for fluorescence microscope experiment). The cardiomyocytes were incubated with DCF-DA for 1 hr at 37°C. The 96-well culture plates was read for fluorescence intensity at λ excitation; 488 nm and λ emission; 510 nm. For 6-well culture plates, cardiomyocytes from each treatment were selected and images were captured using fluorescence microscope. The cardiomyocytes intensity was analyzed using the ImageJ software. The fluorescence intensity was recorded as relative intensity unit (RFU) and analyzed using SPSS 20.0 (International Business Machines Corporation (IBM), USA). For the fluorescence microscope experiment, results are expressed as images and for the microplate experiment, results are expressed in a bar graph.

The Quantigene Plex (QGP) assay (Affymetrix™) was used to measured gene expression. In this part, cardiomyocytes were seeded in 6-well culture plate. After treatment, the cardiomyocytes were detached using trypsin and centrifuged at 1000 rpm for 5 min. The pellets were resuspended in culture medium (without FBS, Pen/Strep, L-Glut) and suspensions were transferred into 96-well culture plates accordingly. Prior to signal amplification, five sets of beads, probe sets, and samples in a total volume of 100 µl were incubated on 96-well hybridization plate. A 100-µl volume of pre-amplifier solution was added to each well and incubation was continued for an hour at 50°C in shaking incubator (Vortemp, USA). Label probe was added after washing. For detection, 100 µl streptavidin phycoerythrin (SAPE) Working Reagent was added and then analyzed using Luminex instrument (Luminex Corp. USA). In this assay, beads were targeted for *caspase-3* and *caspase-9* gene were quantified and normalized with housekeeping *Hprt, Gusb* and *Gapdh* genes. The result of average signals was shown as the mean fluorescence intensity (MFI) for targeted genes and determined for each sample. Gene expressions were presented as fold changes. The normalized genes for the treated samples were divided with value for untreated sample to calculate their fold changes value. Fold changes were presented as either downregulate (< 1), no changes (= 1) or upregulate (> 1).

The caspase-3 and caspase-9 protein expressions were measured using the enzyme linked immunosorbent assay (ELISA) based assay. The micro plate well pre-coated with caspase-3 (Elabscience®, China; E-EL-R0160) and caspase-9 (Elabscience®, China; E-EL-R0163) ELISA kit were used. The cardiomyocytes were treated with UDCA, $CoCl_2$ (hypoxia) and pre-UDCA. Then, treated cardiomyocytes were lysed using the Pierce® RIPA lysis buffer (Thermo Scientific) mixed with protease inhibitor (1×). Proteins were quantified using Bradford assay (Thermo Scientific, USA), a spectroscopic procedure in which the absorbance of the Coomassie dye changes from red/brown to blue (indicate protein concentration). The standard curve of Bovine serum albumin was generated. The OD was measured by the Microplate Reader (Victor X5, Perkin Elmer) at a wavelength of 450 nm. The average of triplicates reading from each standard and samples were subtracted to the average of standard OD (0 ng/ml) to eliminate background noise. The standard curve was plotted for mean OD value for each standard on the y-axis and against concentration on the x-axis. The standard curve of absorbance against concentration was plotted with fitted regression line showing it R-squared value and chart equation (y = mx + c; y = OD value at 450 nm; m = slope; x = concentration; c = intercept of y-axis). The R-squared (r2) was statistical measurement of how close the data lies to the fitted regression line. The sample absorbance (y-value) was extrapolated using the standard curve. The amounts of caspase-3 and caspase-9 protein expressions absorbance were obtained.

3.0 RESULTS AND DISCUSSION

Major modes of cell injury and cell death involve oncosis, necrosis and apoptosis. The apoptosis or necrosis shares similar signaling pathways but different stimulus. The apoptotic signal is important for cells to proliferate and maintain their cell cycles in the complex system of cells' defend mechanism. Mitochondria is the energy producing organelle that directly or indirectly involve in ROS generation [31]and caspases activity [32] in the apoptosis signaling pathway. In cardiomyocytes, apoptosis signaling pathway involve reactive oxygen species (ROS) generation [22] and caspases activation [33]. $CoCl_2$ is chemical that mimic the hypoxic response and promotes cell death in cultured cells. Borenstein et al. (2010) demonstrated that $CoCl_2$ reduces cell viability in murine mammary cancer cells (LMM3) with upregulation of Hif-1a expression [34]. Long term activation of Hif-1a shown to mediate apoptosis dependent of NIP3, p53 protein and caspase activity in hepatoma cell line, HepG2[35]. As reported by others, $CoCl_2$-induced hypoxia was also observed in heart [36], lung [37] and smooth muscle cells [38]. Cobalt (Co^{2+}) accumulation affects myocardium function in the heart with minor increase of left ventricular thickness in population exposes to environmental contamination of cobalt [39].

Adaptation of cardiomyocytes to hypoxia microenvironment is associated with increase of apoptosis inhibitor gene, in between pVHL and GRP78 [40], hypoxia inducible factor (Hif-1alpha) [41], ROS and p38 [42] and release of cytochrome c and caspase 3 activity [43]. Figure 1A shows that $CoCl_2$ significantly increased ROS production after 0.5 hr (30 min; 7.67 a.u ± 1.202; p = 0.47), 1 hr (19.67

a.u ± 1.764; p = 0.003), 6 hr (23.33 a.u ± 1.856; p = 0.003) and 24 hr (31.33 a.u ± 1.202; p = 0.00) of treatment compared to untreated cardiomyocytes (2.33 ± 1.202 a.u). 24 hr $CoCl_2$ treated cardiomyocytes showed the highest production of ROS. Our result suggests that 24 hr $CoCl_2$ and hydrogen peroxide (H_2O_2) treatment significantly increased ROS production in cardiomyocytes. (Figure 1A and 1B). Hydrogen peroxide wasused as the positive control for ROS generation. The results demonstrated is iln line with Carvour et al. (2008) reported that during long term hypoxia, ROS production were highly elevated [59].

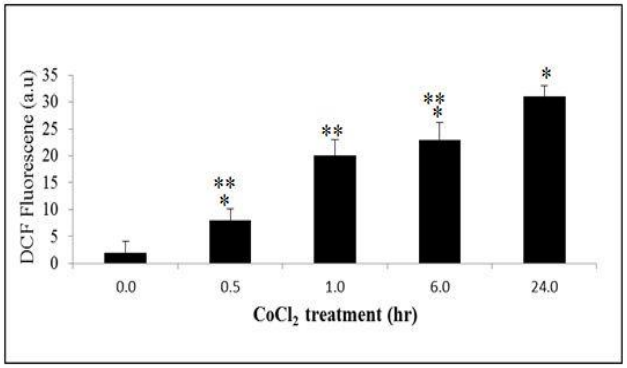


Figure 1A The ROS production in cardiomyocytes treated with $CoCl_2$ at different time point (30 min, 1 hr, 6 hr and 24 hr). (*, indicates significant difference at $p < 0.05$ compared to untreated cardiomyocyte; **, indicates significant at $p < 0.05$ compared to 24 hr, n = 3 independent experiments)

Excess ROS production in the heart or cardiomyocyte leads to expression of apoptotic protein and DNA damage, which subsequently induce cell death. Despite this, low amount of ROS mediates physiological functions such as cell growth, differentiation and metabolism in cardiomyocytes [44]. Therefore, maintaining the ROS production in hypoxic cardiomyocytes is important to promote cell survivability. Duranteau et al. (1998) demonstrated that increase of ROS production was associated with the reduction of heart's contraction [46]. In Figure 2, results showed that pre-UDCA (1.0 ± 0.385 RFU; p = 0.011) significantly reduced ROS production compared to $CoCl_2$ treated cardiomyocytes (1.3 ± 1.453 RFU; p = 0.004). While Post-UDCA (1.1 ± 1.788 RFU; p = 0.100) showed no significant reduction in ROS production compared to $CoCl_2$ treated cardiomyocytes (1.3 ± 1.453 RFU; p = 0.004). The result suggests that pre-UDCA abolished the ROS production in hypoxic cardiomyocytes. Similarly, Rodrigues et al. (1998) reported UDCA inhibition of DCA-induced apoptosis in rat liver. The effect was suggested to be mediated through the reduction of mitochondrial swelling, inhibition of ROS generation and release of Bax protein [47]. During hypoxia, upregulation of caspase-9 and caspase-3 activity was reported to be dependent on upregulation of ROS generation [23].

$CoCl_2$ induces hypoxia in cells by stabilizing the hypoxia-inducible factor (HIF)-a protein as the binding of cobalt (Co^{2+}) to oxygen dependent degradation (ODD) domain of Hif-2a or deactivating proline hydrogenase in cytoplasm [48]. In neuronal cell line, pheochromocytoma-12 (PC12), $CoCl_2$ induced apoptosis was shown to be mediated by an increased of ROS production [49].

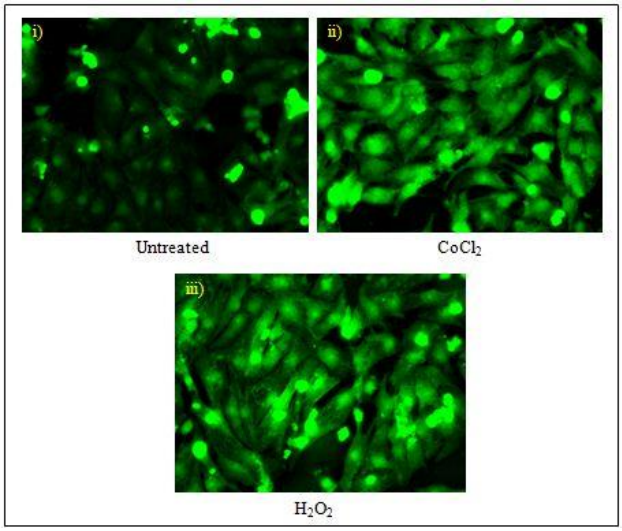


Figure 1B ROS production in untreated, $CoCl_2$ and H_2O_2 treated cardiomyocytes stained with DCF-DA. The cardiomyocytes stained with DCF-DA for (i) untreated, (ii) $CoCl_2$ and (iii) H_2O_2. The changes were viewed under fluorescence microscope (Olympus, USA). n = 3 independent observation

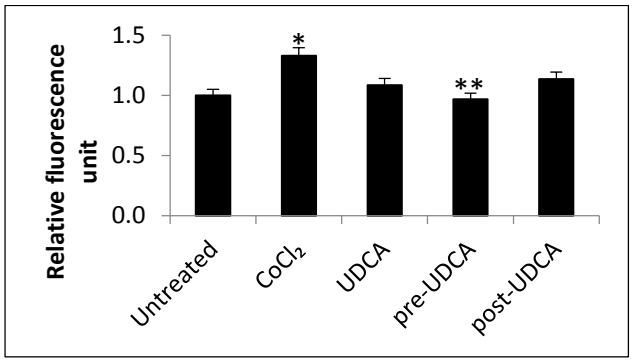


Figure 2 ROS production in cardiomyocytes of $CoCl_2$-induced hypoxia. ROS production increased significantly in $CoCl_2$ (1.3 ± 1.453 RFU; p = 0.004), while those effects were not observed in pre-UDCA cardiomyocytes (significant difference at $p < 0.05$; *, compared to untreated cardiomyocytes, **, compared to $CoCl_2$, result was obtained from 3 independent observation, n = 3).

Caspases were produced as inactive monomeric procaspases which required dimerization or cleavage of its active site for activation. Caspase-9 known as the initiator protein consists of caspase recruitment domain (CARD) which activates caspase-3-induced apoptosis in mouse embryonic

brains [50]. Therefore, caspase-9 is known as an important initiator of caspase-induced apoptosis cascade. In here, $CoCl_2$ treated cardiomyocytes did not showed a significant differences of *caspase*-9 gene(0.60 ± 0.217 fold; p = 0.086), and protein expression (0.97 ± 0.07 OD; p = 0.520), compared to untreated (1.00 ± 0.00 OD) (Figure 3). However, UDCA seems to downregulate caspase-9 protein expression in $CoCl_2$ treated cardiomyocytes (0.75 ± 0.06 OD; p = 0.020) compared to untreated (1.00 ± 0.00 OD) (Figure 3). Meanwhile no changes of caspase-3 were observed at gene and protein level (Figure 4). Downregulation of caspase-9 protein expression could be due to the inhibition of cytochrome c release of the mitochondria-dependent apoptotic pathway [51]. In hypoxia-reoxygenated human lymphocytes ROS activity was shown independent of caspases activity, as incubation with N-acetylcysteine, a ROS scavenger inhibits caspase activation [52]. Apart from that, Moungjaroen et al. (2006) reported that ROS induced cell death in the human lung epithelial cancer cells is mediated by caspase-9 activation and down regulation of Bcl-2 protein [53]. Inhibition of caspase-9 lead to downregulation of apoptotic signals in neuron of rat Parkinson models [54]. However, Kim et al. (2003) demonstrated that ROS production requires activation of caspase-9 but independently promotes cytochrome c release and limit the mitochondria induced-apoptosis in hypoxic injury of human neurogenic brain, SK-N-MC cells [23]. In here, UDCA was shown to downregulates caspase-9 protein expression but no effects on caspase-3 protein was observed in $CoCl_2$-induced hypoxic cardiomyocytes. In contrast, Schoemaker et al. (2004) reported that Taurine conjugated-UDCA protects rat hepatocytes from bile-acid induced apoptosis through inhibition of caspase-3 activity and suppression of pro-apoptotic Bax protein [55]. Downregulation of caspase-9 protein expression could be due to the inhibition of cytochrome C release of the mitochondria dependent apoptotic pathway [51]. Reduction in apoptotic signals such as caspase-9 protects heart from ischemic injury [56]. Phosphorylated Akt reported to inhibit cytochrome C release and block activation of caspase-9 in hypoxic neonatal rat cardiomyocytes [57]. Previously, we have reported that UDCA upregulates survival proteins, Akt and ERK in hypoxic cardiomyocytes [58].

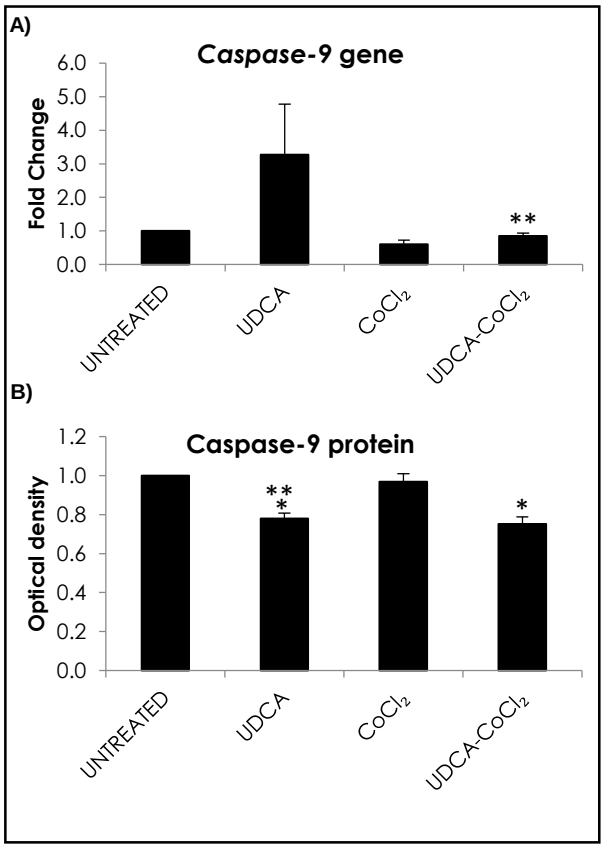


Figure 3 Expression of *caspase*-9 gene and protein in cardiomyocytes of $CoCl_2$-induced hypoxia. Expression of (A) *caspase*-9 gene measured using QGP 2.0 assay; (B) caspase-9 protein measured using ELISA (significant at $p < 0.05$; *, compared to untreated, **, compared to $CoCl_2$, result was obtained from 3 independent experiment, n = 3)

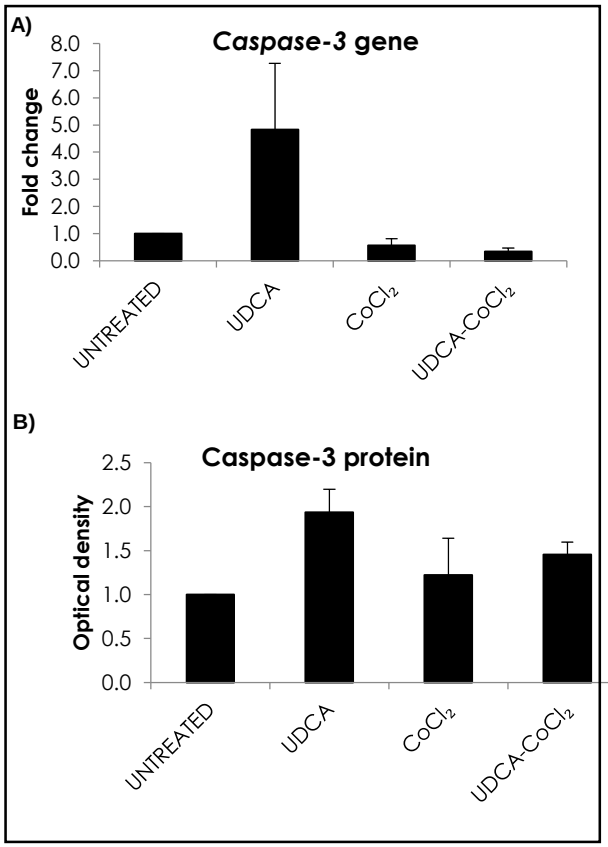


Figure 4 Expression of caspase-3 gene and protein in cardiomyocytes of $CoCl_2$-induced hypoxia.. Expression of (A) caspase-3 gene was measured using QGP 2.0 assay (B) caspase-3 protein measured using ELISA (result was obtained from 3 independent experiments, n = 3)

4.0 CONCLUSION

In conclusion, data obtained from this study suggests that UDCA promotes reduction in ROS generation and downregulates caspase-9 protein expression. This study provides an insight of UDCA as an alternative drug used to promote cardioprotection via regulation of anti-apoptotic signaling in $CoCl_2$-induced hypoxia.

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