Molecular and cellular response of co-cultured muscle and fat cells towards hypoxia condition

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Cobalt chloride ($CoCl_2$) is a well-known hypoxia mimetic agent, and it is inducers of hypoxia-like responses. During hypoxia HIF-1 is activated and it regulates many genes. Therefore, the objective of this study was to investigate the cellular and molecular response of the co-cultured cells during hypoxia condition. Monolayer and co-cultured 3T3-L1 cells were exposed to $CoCl_2$ and a significant induction in HIF1, reactive oxygen species (ROS), lipid peroxides (LPO) and reduction in glutathione (GSH) and catalase were observed. The expressions of pro-apoptotic genes were significantly upregulated, whereas the anti-apoptotic gene, i.e. Bcl2 was down regulated during hypoxia in mono and co-culture 3T3-L1 cells. However, the co-culture of 3T3-L1 cells show significantly higher induction of oxidative stress and apoptotic genes in comparison to moncultured 3T3-L1 cells. The reason may be the communication between the cells and the soluble factors helps in cell survival/death from hypoxia. Because, when the fat and muscle cells grow and differentiate in close vicinity, they interact and communicate via several different pathways, depending on their proximity and mutual ability to interact or communicate. So, the co-culture systems may be useful in evaluating the inter cellular communication and composition of two different cell types.

Key Words: Apoptosis, Co-culture, C2C12, 3T3-L1, Hypoxia**.**

I. INTRODUCTION

Mammalian cells have been developed adaptations for survival from hypoxic condition. Cobalt chloride ($CoCl_2$) is a well-known hypoxia mimetic agent, and it is one of the best chemical inducers of HIF-1 [1]. HIF-1 has an important role and apoptosis prevent cell death, or cell proliferation [2] can be induced by HIF-1. Cobalt chloride ($CoCl_2$) can induce reactive oxygen species (ROS) and a loss of mitochondrial membrane potential (MMP) in different cells.

In muscle cells, hypoxia is an important adaptive stress; it's up-regulates the regulatory subunit of hypoxia-inducible factor-1 (HIF-1) [3]. During severe hypoxia condition the structure of muscle changes, including a clear reduction in muscle fiber area [4]; furthermore, cellular signs of mitochondrial degradation products overcome under conditions of increased reactive oxygen species (ROS) formation [5].

To study the natural interaction between populations co-culture system has used. Co-cultures are very important for drug research because they provide a more representative in vivo-like tissue model [6]. The process of muscle development starts earlier than the fat in fetal, postnatal, and adult animals. The developing fat cells closed to muscle undergo all growth and differentiation phases in close proximity, similar to mature, multinucleated, and functional skeletal muscle [7]. The aim of the present investigation was to study the $CoCl_2$ modulation at the level of gene product implicated in hypoxia (HIF1α) and apoptotic genes in co-culture system.

II. MATERIALS AND METHODS

Mouse myoblast C2C12 and mouse pre-adipocyte 3T3-L1 cell lines used in the study were procured from the American Type Culture Collection (ATCC), Manassas, USA and maintained at Department of Animal Science and Biotechnology, Chonbuk National University, Jeonju, Korea Republic of, as per the standard protocols. Cytotoxicity assessment of $CoCl_2$ was done using standard endpoint, i.e. tetrazolium bromide MTT assay. The 3T3-L1 cells were differentiated into adipocytes with the help of differentiation medium. The cells fully differentiated in to adipocyte between 7 to 15 days after induction, as evidenced by observation of lipid droplet formation by Oil Red O staining. Cells were co-cultured using transwell inserts with a 0.4-μm porous membrane to separate 3T3-L1 and C2C12 according to the protocol of Sun and Zemel 2008 [8]. Following cell differentiation, inserts containing C2C12 were transferred to 3T3-L1 plates. Following co-culture for

12, 24 and 48 h, the 3T3-L1 cells in the lower wells were harvested for oxidative stress and apoptosis study.

III. RESULTS AND DISCUSSION

The 3T3-L1 cells were fully differentiated in to adipocyte between 7 to 15 days after the induction, as evidenced by observation of lipid droplet formation by Oil Red O staining. The images of cells on the plate were taken in PBS, shown in figure 1.

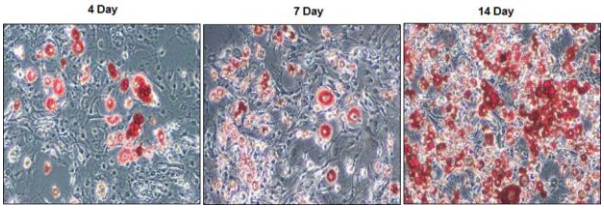
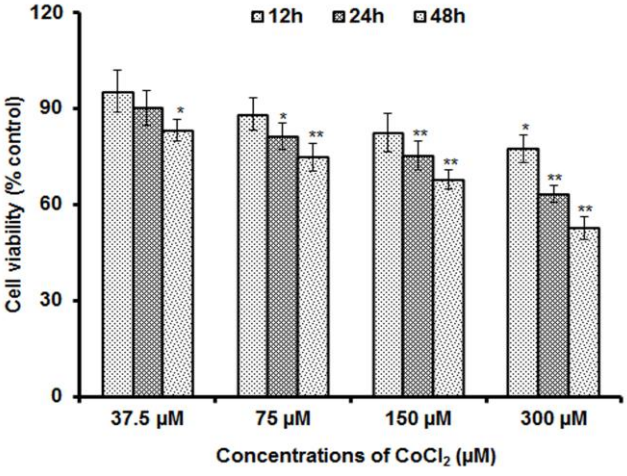


Figure 1: Differentiation of pre-adipocyte cells (3T3-L1) to adipocyte cells. After co-cultured, 3T3-L1 preadipocytes were induced to differentiation, and the inverted microscope images of day 4, day 7 and day 14 (oil red O-stained) were shown, and the cell triglyceride accumulation was detected.

Results of MTT assay are summarized in Fig. 2. C2C12 and 3T3-L1 cells responded to $CoCl_2$ in a dose and time dependent manner. There was no significant reduction in percent cell viability reported all through the exposure period, i.e., till 48 h in the used concentration i.e., 37.5 and 75 µM of the $CoCl_2$. Whereas, the concentrations of $CoCl_2$ used, i.e., 150 and 300 µM were found to cause a gradual reduction in percent cell viability, which reaches to significant levels at and above the exposure period of 24 h (Fig. 2).

Figure 2: Identification of non-cytotoxic dose of cobalt chloride



($CoCl_2$) in C2C12 and 3T3-L1 cells. Cells were exposed to $CoCl_2$ (37.5-300 µM) for 12–48 h in C2C12 (A) and 3T3-L1 cells (B). The percent cell viability was assessed using MTT assay. Values are given as mean ± standard error of the data obtained from three independent experiments. * = $p<0.05$, ** = $p<0.01$.

A significant increase in ROS and LPO and decrease in the level of GSH and catalase was recorded after the exposure of $CoCl_2$ at each time point. The level of ROS and LPO were less and the level of GSH and catalase were high in co-cultured 3T3-L1 cells in comparison to monolayered 3T3-L1 cells (Fig. 3).

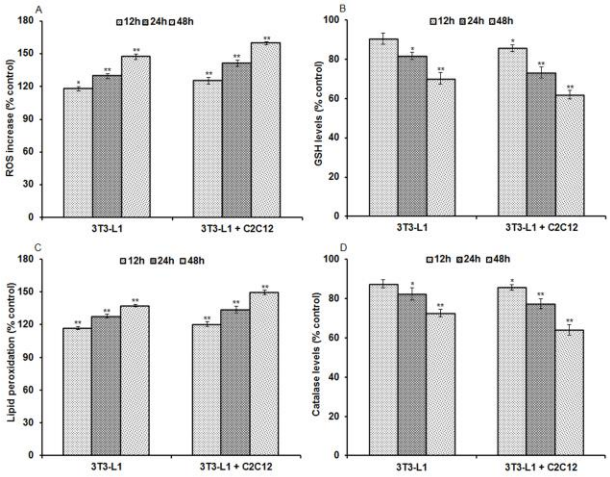


Figure 3: Oxidative stress study in mono and co-cultured 3T3-L1 cells. Percent change in ROS generation (a), GSH activity (b), Change in levels of lipid peroxidation (c) and catalase activity (d) after the exposure of 150 µM of CoCl2 for 12, 24 and 48h time periods assessed by the micro plate reader. Data represented are mean±SE of three identical experiments made in three replicate. *$p<0.05$ and ** = $p<0.01$ in comparison to respective unexposed controls.

Several pathologies as well as many cellular processes have been affected after the decrease in the level of oxygen [9]. ROS act as an O_2 sensor and it modulates the gene expression in response to hypoxia [10]. Continuous hypoxia would result in oxidative stress and ROS may be associated with the induction of apoptosis [11]. Several studies show that the ROS production directly affects the mitochondrial function and inducing mitochondrial membrane potential in various cells [12]. GSH plays important role in protecting cells from oxidative damage and maintaining redox homeostasis. It is synthesized in the cells in abundance and known to be affected by $CoCl_2$ exposure [13]. Though, we observed depletion of GSH in both mono and co-cultured cells receiving $CoCl_2$, but the magnitude of depletion was statistically greater in the monocultured cells in comparison to co-cultured cells. So, the depletion of GSH levels in both mono and co-cultured cells observed in the present investigation could be attributed to the increased utilization of GSH-GPx in detoxification of H2O2 generated by $CoCl_2$. But,

increased production of ROS inhibits the mitochondrial respiration and known to induce progressive lipid peroxidation [14]. In the present investigations, a significant high level of LPO was observed in monocultured cells than the co-cultured cells after the exposure of $CoCl_2$.

The significant up regulation of hypoxia inducible factor (HIF1α), pro-apoptotic genes bax, p53, caspase-3 and caspase-9 and down regulation of anti-apoptotic gene bcl2 have been recorded after the exposure of $CoCl_2$ in both monocultured and co-cultured 3T3-L1 cells. The co-cultured cells show higher expression of apoptotic genes in comparison to monocultured 3T3-L1 cells (Fig. 4). Several studies have shown that the mitochondrial membrane permeabilization has been promoted by the interaction of cytosolic p53 protein with mitochondria and plays a key role in the regulation of apoptosis [15]. The literature has shown that the cytosolic p53 protein induces the pro-apoptotic members of the Bcl2 family, such as Bax and its displacement with anti-apoptotic Bcl2 protein [16]. However, the induction of nuclear p53 protein preventing genotoxicity by inducing transcriptional reprogramming, which finally leads to controlled cell death [17]. Thus, the alterations in the expression profile of marker genes in this study indicate that cytosolic p53 triggers the mitochondrial apoptotic cascade in both types of cultured cells after the exposure of CoCl2.

IV. CONCLUSION

Our study demonstrates that the exposure of $CoCl_2$ significantly induces the expression of hypoxia inducible factor (HIF1). Due to hypoxia condition, ROS generation increases, which initiates oxidative damage in the cells. The increased ROS levels upregulates p53 and diminish Bcl2 protein, leading to a deteriorated ratio of Bcl-2/Bax which results in the release of cytochrome-c.

Finally, the cytochrome-c activates caspase-9 and subsequently triggers the caspase-3 cascade leading to apoptosis in both type cultured cells. However, the co-cultured cell responses significantly high towards the hypoxia induced oxidative stress. While, the monocultured cells also showing the oxidative stress towards the $CoCl_2$ exposure, but the magnitude is lower in comparison to co-cultured cells.

With the advent of 3D cell cultures, in vitro studies are now closer to animal models in many aspects. They offer biologically superior structures which can be utilized to study complex interactions that were not possible with 2D cultures. 3D cultures can efficiently be used to understand complex studies for basic and applied research. The dynamic advancements in the instrumentation technology and material sciences have augmented the parallel increase in complexity in cell culture techniques. 3D cell cultures have a myriad of applications; for drug discovery, pharmacological

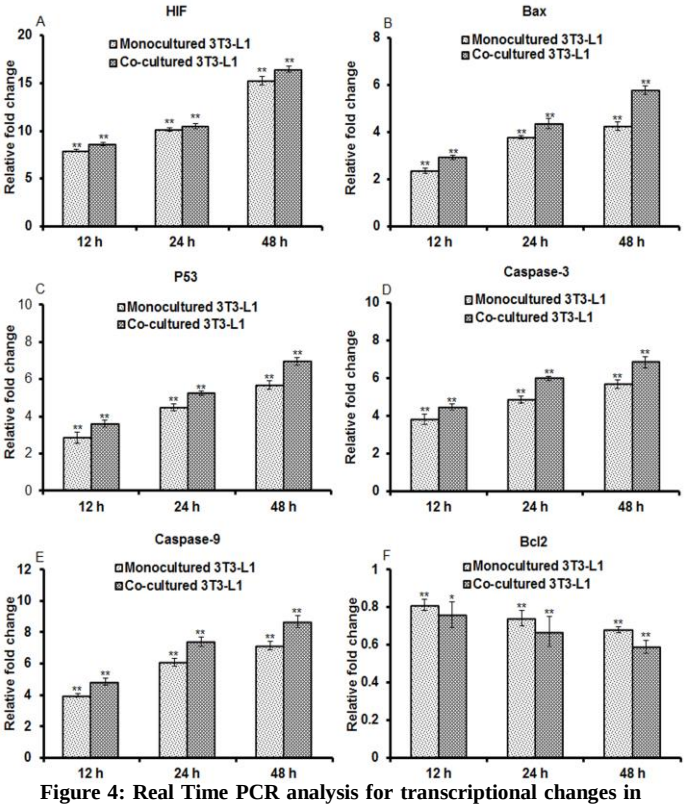


Figure 4: Real Time PCR analysis for transcriptional changes in apoptotis genes in monocultured and co-cultured 3T3-L1 cells. Fold changes in altered mRNA expression of apoptotic genes in monocultured and co-cultured 3T3-L1 cells following the exposure of $CoCl_2$ (12-48 h). β-actin was used as an endogenous control to normalize the data and CoCl2 induced alterations in transcripts are expressed in fold changes (mean ± standard error) compared with unexposed controls. * = $p<0.05$ and ** = $p<0.01$ in comparison to respective unexposed controls.

studies, understanding cell physiology, gene and protein expressions, cancer research, tissue engineering and also for increasing the biotech industry productivity. The co-culture system provides fundamental information to reveal differences between cell-to-cell effects and alteration of discrete pathways and to know the regulation of fat and muscle deposition in meat animals. The co-culture system is developed to address

the migration regulation, proliferation, differentiation of satellite cells by circulating cells, tissue engineering and also for increasing the biotech industry productivity. Co-culture system facilitates and enhances the definition of the specific factors responsible for communication between two different muscle cell types.

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