



## **HYPOXIA PROMOTES GROWTH AND VIABILITY OF HUMAN ADIPOSE-DERIVED STEM CELLS WITH INCREASED GROWTH FACTORS SECRETION**

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### **ABSTRACT**

*Adipose-derived stem cells (ASCs) have been found residing in a native microenvironment with low oxygen tension (i.e hypoxia) in human body. Oxygen tension plays a crucial role in regulating their function. The aim of this study is to investigate the effects of hypoxia on proliferation and survival rate of ASCs. Human ASCs were cultured at 21% O<sub>2</sub> (normoxia) or 2% O<sub>2</sub> (hypoxia) for up to three passages. We found that ASCs displayed a higher proliferation rate under hypoxia as compared to normoxia. The viability of ASCs was higher in hypoxia than normoxia without altering their characteristics. These properties might be correlated with the up-regulation of HIF-1 $\alpha$  and the*

*increased transcriptional and translational level of growth factors, VEGF-A and bFGF. Taken together, 2% oxygen tension has been suggested as an ideal condition for expansion of ASCs efficiently, where large number of cells can be produced over a short period of time for clinical use.*

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**Keywords:** Adipose-derived stem cells, Hypoxia, Characteristics, Proliferation, Viability, HIF-1 $\alpha$ , Growth factors.

## 1. INTRODUCTION

In recent years, the application of stem cells in medical therapy has drawn worldwide attention. Their ability of self-renewal and potential of differentiating into various cell types make them a promising cell source for regenerative medicine [1]. Currently, new attention has been focused on the use of mesenchymal stem cells (MSCs) in clinical therapy, suggesting that these cells may produce a better therapeutic effect [2, 3]. MSCs were first characterized in bone marrow [4]. Subsequently, they were found in nearly all adult tissues such as adipose tissue [5], synovium [6], skin [7], umbilical cord blood [5], skeletal muscle [8] and deciduous teeth [9].

Among various types of MSCs, adipose derived stem cells (ASCs) have become an attractive source for cell therapy due to their relative abundance and readily accessibility [10]. With increasing incidence of obesity in recent years, adipose tissue can be easily obtained through liposuction or abdominoplasty surgery [11]. Interestingly, ASCs have been found residing in their native niche with low oxygen tension to ensure the maintenance of their characteristics. Researchers reported that the oxygen level of less than 4% represents the physiological condition of native adipose tissue [12, 13].

At present, most studies are performed under 21% O<sub>2</sub> which represents a non-physiological atmospheric oxygen tension. While there has been controversy in several research findings and literature, the effects of hypoxia on ASCs has yet to be determined. Many studies have demonstrated that hypoxia is more beneficial for proliferation and survival of human ASCs as compared to normoxia especially at oxygen tension as low as 2% [14, 15]. It has been suggested that under hypoxia, the upregulation of HIF-1 $\alpha$  increased the expression of a variety of growth factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF), hepatocyte growth factor (HGF) and platelet-derived growth factor (PDGF) which might have a positive impact on cell survival and growth [16-18].

In this study, besides the effects of 2% O<sub>2</sub> on cell properties, we investigated the role of HIF-1 $\alpha$  and several growth factors in growth and survival of ASCs. We first characterized the ASCs by their morphology, expression of cell surface markers and differentiation potential. Subsequently, we investigated their viability and proliferation rate, followed by determining the expression level of HIF-1 $\alpha$  and the secretion of growth factors under this physiological condition.

## 2. MATERIALS & METHODS

### 2.1. Isolation and Culture of Human ASCs

Human adipose tissue was collected from healthy donors aged 25-45 years after Caesarean section, with the approval of the Medical Ethics Committee University Malaya Medical Centre (reference no. 996.46). Adipose tissue was washed twice with phosphate buffered saline (PBS) (Sigma-Aldrich, USA) before mincing. The tissue was then digested with 0.3% collagenase type I (Worthington, USA) at 37°C with 250rpm shaking for 30 minutes. Afterwards, the mixture was centrifuged and the pellet obtained was washed and cultured in T-25 tissue culture flasks with complete culture medium, Dulbecco's Modified Eagle's Medium (DMEM)/Ham F-12 medium consisting of 10% FBS, 1% glutamax, 1% antibiotic-antimycotic and 1% vitamin C (Gibco, USA). As a control, the flasks were placed at 37°C in CO<sub>2</sub> incubator containing 21% O<sub>2</sub> and 5% CO<sub>2</sub>. Hypoxic cultures were kept at 37°C under 2% O<sub>2</sub> and 5% CO<sub>2</sub> in O<sub>2</sub> control incubator (Galaxy 170 R, New Brunswick Scientific, USA) supplied with N<sub>2</sub>. Media were replaced 3 times a week. ASCs were sub-cultured before reaching 90% confluency and passage 3 cells were used for all the experiments unless otherwise indicated.

### 2.2. Immunophenotyping Characterization

To characterize mesenchymal stem cells (MSCs), three standard criteria proposed by [Dominici, et al. \[19\]](#) was followed. Firstly, MSCs must be plastic-adherent. Secondly, they must be able to express their major surface markers CD73, CD90 and CD105 and lack expression of haematopoietic markers CD14, CD34 and CD45, B cell marker CD 19, and histocompatibility class II cell surface receptor HLA-DR, DP, DQ. Thirdly, MSCs must have the ability to differentiate into adipocytes, osteoblasts and chondroblasts.

Besides examining the morphology of ASCs under normoxia and hypoxia, immunophenotyping characterization of the cells was performed. Briefly, the cells were harvested and resuspended in PBS. Subsequently, the cells were incubated in the dark for 20 minutes with surface markers antibodies. The antibodies used were CD73-PE, CD90-FITC, CD105-FITC, CD14-PE, CD19-PE, CD34-FITC, CD45-FITC, HLA DRDPDQ-FITC and their specific isotype controls IgG1-FITC, IgG2-FITC, IgG1-PE and IgG2-PE (Becton Dickinson, USA). A minimum of 10000 cell events were collected for sample analysis by BDFACS Canto II flow cytometer (Becton Dickinson) and data analysis was performed using FlowJo analysis software (Treestar, USA).

### 2.3. Differentiation Ability

Both hypoxic and normoxic cultured ASCs were induced to differentiate towards adipogenic, osteogenic and chondrogenic lineages. The cells were cultured in their appropriate differentiation medium for 3 weeks to be directed to respective lineages differentiation prior to staining. The media was changed every 3 days. Adipogenic differentiation was performed by culturing ASCs in petri dishes with adipogenic differentiation medium consisting of high glucose DMEM supplemented with 10% FBS (Gibco), 1 µM dexamethasone, 0.5 µM isobutyl-1-methyl xanthine, 10 µM insulin and 200 µM indomethacin (Sigma-Aldrich). Oil red O stain (Sigma-Aldrich) was used to assess the adipogenic differentiation by staining the lipid droplets of adipocytes.

Osteogenic differentiation was induced by seeding the cells in petri dishes with osteogenic differentiation media comprised of high glucose DMEM with 10% FBS (Gibco), 10 mM  $\beta$ -glycerophosphate, 0.05 mM ascorbic acid-2-phosphate and 100 nM dexamethasone (Sigma-Aldrich). After the fixation and washing with deionized water, Alizarin Red stain (Sigma-Aldrich) was used to detect the formation of calcium deposits.

For chondrogenic differentiation, the cell pellets were cultured in chondrogenic medium comprised of complete culture media DMEM/F12 supplemented with 1% FBS, 1% vitamin C, 1% glutamax, 1% antibiotic-antimycotic (Gibco), ITS premix (Becton Dickinson), 100nM dexamethasone, 50  $\mu$ g/ml ascorbate- 2-phosphate, 40 $\mu$ g/ml L-proline (Sigma-Aldrich), 10 ng/ml TGF- $\beta$ 150 ng/ml, IGF-1 (Peprotech, USA). After 3 weeks of culture, the pellets were fixed in 10% formalin (Sigma-Aldrich) and then following embedding, sectioning and all the standard histological procedure. After that, proteoglycan was detected by alcian blue stain (Sigma-Aldrich). After the sections were mounted with DPX mounting medium (Sigma-Aldrich), staining was examined under the light microscope (Nikon Eclipse TS100, USA).

#### 2.4. Proliferation Assay

Proliferation rate of ASCs was determined by generating a growth curve. At the third passage, ASCs were seeded in 24 well culture plate with complete culture media at  $5 \times 10^4$  cells per well and were placed under normoxia and hypoxia. On day 1, 3, 7, 10 and 14, the cells from each three wells were detached and counted using a haemocytometer. Cell growth was measured using trypan blue exclusion assay and the number of viable cells was determined at selected time points.

#### 2.5. Apoptosis Assay

Apoptosis assay was carried out to determine cell viability using Annexin V: FITC Apoptosis Detection Kit I (Becton Dickinson) according to the manufacturer's instructions. ASCs were analyzed by flow cytometry using *BD Accuri*<sup>TM</sup> C6 (Becton Dickinson).

#### 2.6. RNA Extraction, DNA Synthesis and Quantitative Real-Time Polymerase Chain Reaction (qPCR)

Each sample was homogenized in TRI reagent (Ambion, USA) and RNA was extracted following the manufacturer's instructions. Reverse transcription was carried out using high capacity RNA-to-cDNA kit (Applied Biosystems). Subsequently, quantitative real time PCR was assessed using *TaqMan* gene expression assays (Applied Biosystems).

The genes tested include HIF-1 $\alpha$  (Hs00153153\_m1), VEGF A (Hs00173626\_m1), bFGF (Hs00266645\_m1), PDGF (Hs00234994\_m1), IGF (Hs01547656\_m1) and HGF (Hs00300159\_m1). The polymerase activation at 95°C for 20 s was followed by 40 cycles of denaturation at 95°C for 1 s, annealing and elongation at 60°C for 20 s. Normalization was performed using housekeeping gene, GAPDH. As a control group, the expression level of normoxia was normalized to 1. Fold changes in gene expression relative to the control were determined.

### 2.7. Enzyme-linked Immunosorbent Assay (ELISA)

To determine the protein secretion of VEGF-A and bFGF, ELISA was performed using human Quantikine ELISA kit (R&D Systems, USA) in accordance to the manufacturer’s instructions.

### 2.8. Statistical Analysis

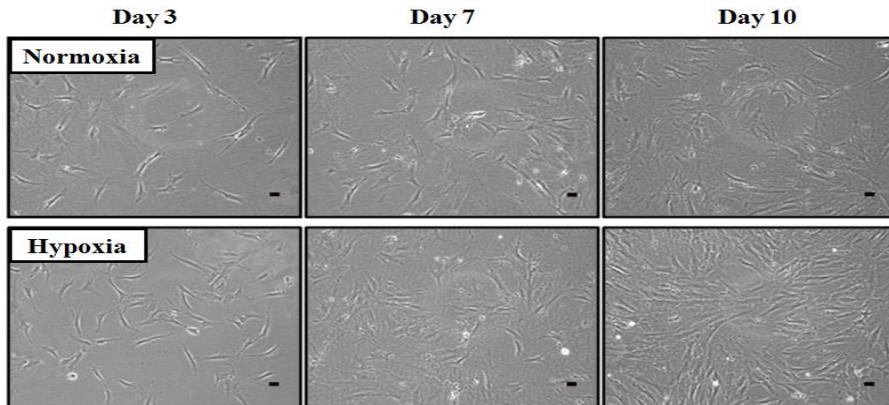
All the experiments were repeated at least three times. Results were presented as mean  $\pm$  standard error of mean (SEM). Comparison between normoxia and hypoxia was determined by Student’s t test.  $p < 0.05$  was considered statistically significance.

## 3. RESULTS & DISCUSSION

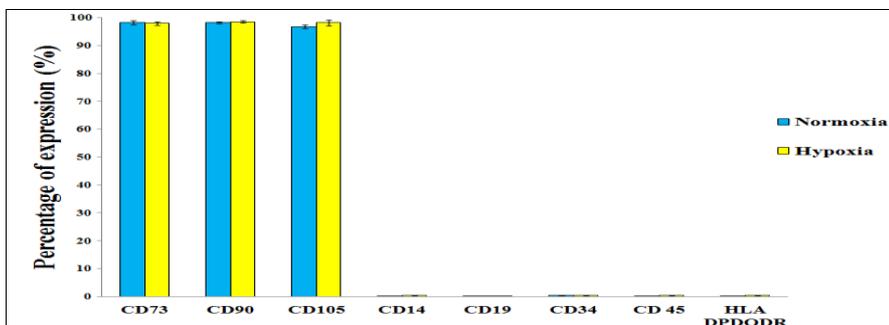
### 3.1. Characterization of ASCs

As ASC is a type of MSC, the cells used in this study were first characterized to prove that they were MSCs [19]. We found that ASCs under normoxia and hypoxia were able to maintain their plastic adherent properties and fibroblast-like morphology (Figure 1). They positively expressed the major MSCs surface markers CD73, CD90, CD105 and were negative for non-MSCs surface markers CD14, CD34, CD45, CD19 and HLA-DR, DP, DQ (Figure 2). In addition, they possess the ability to differentiate into adipocytes, osteoblasts and chondroblasts under both culture conditions (Figure 3). Based on these properties, these cells were defined as MSCs.

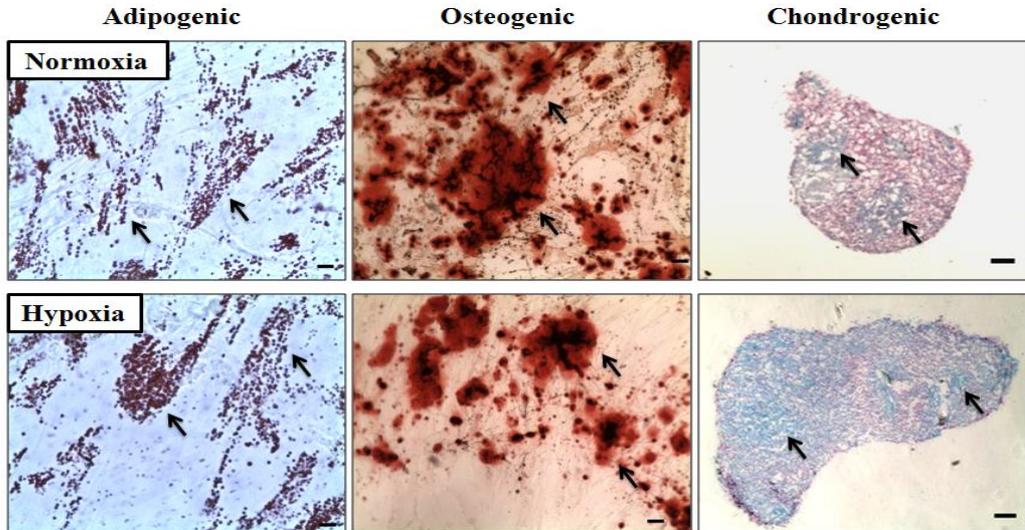
**Fig-1.** Morphology of ASCs under normoxia and hypoxia (Magnification 100x)



**Fig-2.** The expression level of ASCs surface markers under normoxia and hypoxia



**Fig-3.** The multilineage differentiation capability of ASCs under normoxia and hypoxia [Arrows indicate lipid droplets of adipocytes (Magnification 400x), calcium deposits of osteoblasts (Magnification 100x) and proteoglycans of chondroblasts (Magnification 40x)]

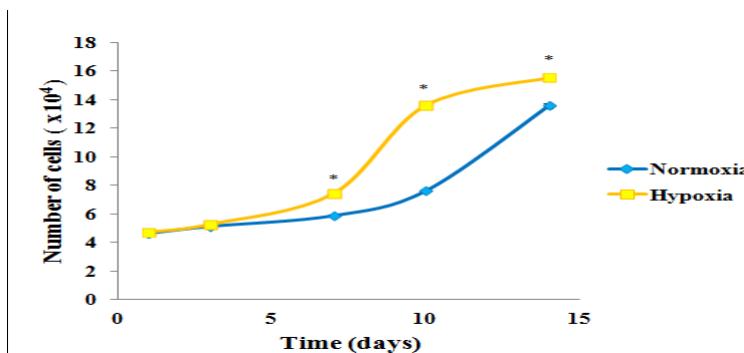


### 3.2. Effect of Hypoxia on ASCs Proliferation

Morphologically, ASCs showed a marked increase in cell density under hypoxia from day 7 of culture in comparison with normoxia (Figure 1). To confirm the effect of hypoxia on proliferation rate of ASCs, growth curve study was performed. The growth curve showed that there was a significant increase in cell number from day 7 of the culture. On day 10, in particular, the number of cells cultured under hypoxia showed 1.79-fold greater ( $13.6 \times 10^4 \pm 0.2 \times 10^4$ ) than normoxia ( $7.6 \times 10^4 \pm 0.1 \times 10^4$ ) (Figure 4).

The higher growth rate of hypoxic cultured ASCs showed that they grew more rapidly under hypoxia than normoxia. Our data suggests that this physiological condition provides more favourable environment for the growth of ASCs. This result was consistent with other findings which demonstrated an increased ASCs expansion rate at 2% O<sub>2</sub> [14, 15].

**Fig-4.** The expansion rate of ASCs under normoxia and hypoxia (Values are mean  $\pm$  SEM, \* p < 0.05 vs normoxia)

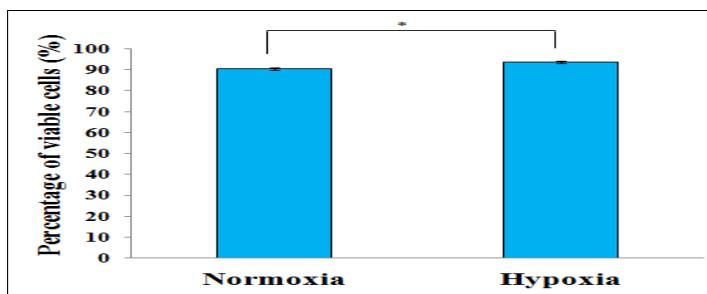


### 3.3. Effect of Hypoxia on ASCs Viability

To determine the viability of ASCs under two different culture conditions, apoptosis assay was performed. Flow cytometry analysis revealed that the percentage of viable ASCs in hypoxia was significantly higher ( $93.77 \pm 0.55\%$ ) than that of normoxia ( $90.4 \pm 0.61\%$ ) (Figure 5), suggesting that low oxygen tension enhanced ASCs viability.

Apoptosis is a process of programmed cell death in response to cellular stresses which leads to the removal of undesired cells from the body [20]. Many studies have reported that hypoxia increased ASCs viability with reduced apoptotic cells as compared to normoxia [14, 21]. The higher cell viability under hypoxia might be due to the lower reactive oxygen species (ROS) production which reduce the rate of cellular apoptosis as compared to normoxia [22].

**Fig-5.** The viability of ASCs under normoxia and hypoxia (Values are mean  $\pm$  SEM, \*  $p < 0.05$  vs normoxia)

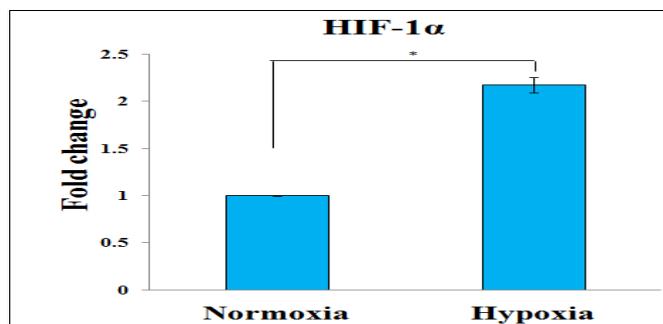


### 3.4. Effect of Hypoxia on HIF-1 $\alpha$ , VEGF and bFGF Expression

To determine if the HIF-1 $\alpha$  and growth factors involve in growth and survival of ASCs, gene expression study was performed. We found that hypoxia showed a 2.17-fold increase in the expression level of HIF-1 $\alpha$  when compared to normoxia (Figure 6).

Generally, HIF-1 $\alpha$  represents one of the subunits of HIF-1, a heterodimeric transcription factor which regulates cell survival and growth under low oxygen condition [23]. The higher expression level of HIF-1 $\alpha$  with an increased proliferation and viability of ASCs under hypoxia provides the evidence that HIF-1 $\alpha$  plays an important role in regulating the cell proliferation and viability under their physiological condition.

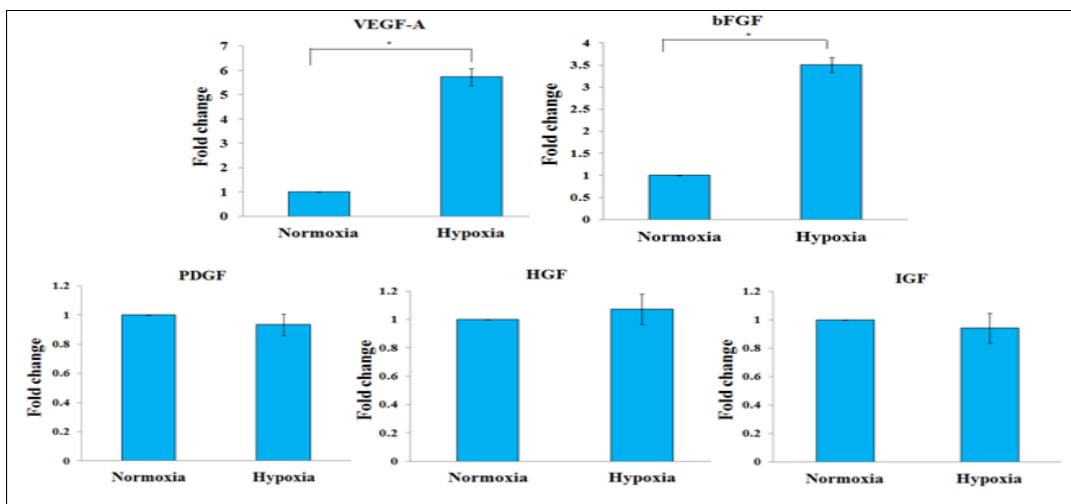
**Fig-6.** The gene expression level of HIF-1 $\alpha$  under normoxia and hypoxia (Values are mean  $\pm$  SEM, \*  $p < 0.05$  vs normoxia)



Besides that, we found there was a 5.73-fold and 3.51-fold increase in the expression of VEGF-A and bFGF, respectively, under hypoxia as compared to normoxia. However, unlike VEGF-A and bFGF, there were no significant differences in the expression level of other growth factors PDGF, IGF and HGF between two different culture conditions (Figure 7). In fact, VEGF-A has been reported to have a very potent mitogenic characteristic. It has been known to be an important paracrine signalling molecule in stem cell-mediated angiogenesis, growth and survival [16]. bFGF regulates a wide range of cellular processes in various cell types. It acts as a potent mitogen and play a major role in proliferation [24]. Other growth factors such as PDGF, HGF and IGF also appear to be necessary for MSCs self-renewal, proliferation, survival and pluripotency maintenance [25].

In present study, an increased expression of VEGF-A and bFGF showed that these growth factors may contribute to the increased proliferation and viability of ASCs. However, the absence of significant differences in the expression of PDGF, HGF and IGF may indicate their minor effect on cell growth and survival. These results were in agreement with the findings of [26], suggesting that hypoxia induced the up-regulation of VEGF-A and bFGF when ASCs were exposed to low oxygen tension which might be involved in regulating the cell growth.

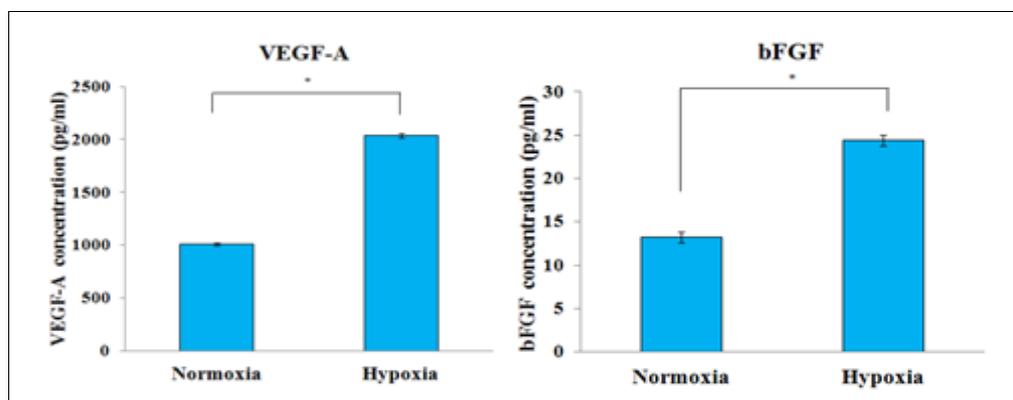
**Fig-7.** The gene expression level of growth factors under normoxia and hypoxia (Values are mean  $\pm$  SEM ,\*  $p < 0.05$  vs normoxia)



### 3.5. Effect of Hypoxia on VEGF and bFGF Secretion

In line with the result of gene expression, ELISA showed that hypoxia had a significant 2.02-fold increase in the secretion of VEGF-A as compared to normoxia. Similarly, the secretion of bFGF was significantly increased 1.84-fold in hypoxia (Figure 8). This result further confirmed the increased production of VEGF-A and bFGF under hypoxia.

**Fig-8.** The concentration of VEGF-A and bFGF secreted under normoxia and hypoxia (Values are mean  $\pm$  SEM, \*  $p < 0.05$  vs normoxia)



#### 4. CONCLUSION

The present study demonstrates that 2% O<sub>2</sub> enhanced viability and growth of ASCs, where HIF-1 $\alpha$  and growth factors, VEGF-A and bFGF might play a crucial role in regulating these activities. Since 2% O<sub>2</sub> appears to be a suitable physiological condition for promoting ASCs proliferation and viability, further investigation of this physiological oxygen tension on potential of ASCs to differentiate into various types of cells is essential to determine their therapeutic potential and possible clinical impact. Taken together, our findings suggest that growing ASCs at 2% oxygen tension may be an ideal option for an efficient cell expansion for clinical therapy.

#### 5. ACKNOWLEDGEMENT

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