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IN VITRO ANALYSIS AND DIFFERENTIAL CHARACTERISATION OF NUCLEUS PULPOSUS DERIVEDMESENCHYMAL STEM CELLS AND ITS ASSOCIATED STEMNESS AND GENE EXPRESSION CHANGES SUBJECTED TO INFLAMMATORY MARKERS IN HYPOXIC AND NORMOXIC CULTURE CONDITION

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ABSTRACT

Degenerative disc disease (DDD) is generally considered as a chronic, progressive and painful disease that is described by inflammation and breakdown of tissue within the intervertebral disc. In recent days, nucleus pulposus mesenchymal cells (NPMSCs) have been proved to be a successful in producing the intended result in therapy of degenerative disc disease (DDD). The aim of the present study was to evaluate the biological characteristics analysis of normal and degenerative nucleus pulposus derived mesenchymal stem cells and also the role of inflammatory cytokines on mouse derived NPMSCs and its related gene expression in normoxic and hypoxic culture setting. NP Tissues were collected from the mouse and expanded in cell culture in normoxic and hypoxic culture setting, NP derived mesenchymal stem cells phenotypic characterization, Cell cycle and apoptosis was confirmed with Flowcytometry. The multipotent differentiation potential of NPMSCs was analyzed with immuno histochemistry and its specific gene expression analysis was confirmed by gel electrophoresis and reverse transcriptase PCR. NPMSCs from non - degenerative and degenerative mice nucleus pulposus shows the similar phenotypic characteristics, But the expression of markers like stemness, differentiation, chemotactic migration and colony formation was high compared to the degenerative NPMSCs. Here pro-inflammatory markers suppress the expression of hypoxia resistant genes in both normoxic and hypoxic culture environments. Therefore the pro-inflammatory cytokines plays vital role in maintenance of biological behavior of NPMSCs. The findings of this study suggest that NPMSCs shows better proliferation in normoxic and hypoxic culture environment, though the NPMSCs showed the good proliferation in both types of culture condition in the presence of pro-inflammatory cytokines it's proliferation, multipotent differentiation apoptosis highly increased.

Keywords: Disc Degenerative Diseases, Flowcytometry, Glucose Transporter 1, Glucose Transporter -3, Hypoxia Inducible Factor, Intervertebral Disc, Matrix Metallopeptidases, Mesenchymal Stem Cells, Nucleus Pulposus, Propidium Iodide.

1. INTRODUCTION

Intervertebral disc degeneration is mostly considered as the prime reason for disc degenerative disease causes of heavy economic burden in society [1, 2]. Generally, Intervertebral discs (IVDs) consist of three major structures, those are the centrally situated nucleus pulposus (NP), outer annulus fibrosus (AF) and cartilage end plates (CEP). The central nucleus pulposus is composed of nucleus pulposus Cells (NPCs), proteoglycan (PG) and then surrounded by the extracellular matrix (ECM), including type II collagen and aggrecan and cardiac endplates. The outer AF is mainly composed of collagen fibres. NP plays a vital role in maintaining the physiological role of the discs in the process of distribution of the mechanical pressure, which is present on the spine [3, 4]. Intervertebral discs are anatomically considered as large avascular in nature, and also aneural with only sparse nerves and blood vessels present. Recently, many evidences show that the degeneration of the IVD is mainly happening due to the depletion in ECM synthesis, NP Cell loss and increased production of type I collagen [4]. Hence the regeneration capacity and self repair of the disc are limited and also treating thr injured IVDs is a challenging process. The

progression of IVD degeneration is generally happening due to the inability of NP cells to regulate the normal homeostatic tissue remodelling and then finally it leads to low back pain. However, the mechanism behind the IVD degeneration remains largely unknown.

Intervertebral disc degeneration (IDD) is considered as the fundamental basis of back pain, urgently needs more effective therapies instead of symptomatic relief [5]. The quantity of individuals suffering with IDD is anticipated to grow exponentially because of the population ages and it imposes a significant burden on healthcare resources within the close to the future [6]. Though the stem cells (SCs) show the capabilities for self-renewal and multi lineage differentiation [7], generally never survive in harsh environments of IDD, which has strong interaction with hypoxia, acidic pH, limited nutrients and hyper osmolarity [8]. Hence, now a days the regeneration potential of nucleus pulposus progenitor cell and NP derived mesenchymal stem cells, gaining more attention for therapeutic application of IDD.

Disc degeneration and back pain is mainly happening due to some alterations, which takes place in the composition and properties of nucleus pulposus (NP) and its associated mechanical stress and physiological loads [9]. Also numerous biochemical changes, including expanded collagen I and decreasing large proteoglycans (PG) ratio and increasing the ratio of small to large proteoglycans leads to more fibrous NP tissue. The natural repair of degenerated NP is difficult, because of the NP cells is fully depends on nutrient diffusion from the adjacent endplates [10].

Nucleus pulposus is generally considered as a jelly like substance, which is present in the middle of the intervertebral disc. Most important function of these nucleus pulposus is to distribute hydraulic pressure under compressive loads in all directions within each disc. Nucleus pulposus mainly contains proteoglycans, aggrecans, collagen fibrils and Nucleus pulposus cells and reside in the environment, which has limited vascular supply and also it can generate energy through anaerobic glycolysis. The Nucleus pulposus cells produce collagen II, X and also expresses hypoxia inducible factor-I [11] and secrete interleukins 1,6,10 and granulocyte macrophage colony stimulating factor [12]. One of the essential functions of Nucleus pulposus is to promote cell proliferation during mechanical stress.

NP cells is normally adapting to survive in the hypoxic environment through the continuous expression of hypoxia inducible factor (HIF-1α) [4]. It is a transcription factor responsible for resisting the oxygen tension. HIF is normally helix-loop-helix structure with two subunits like α and β. Here both α and β subunits undergo the degradation through oxygen dependent and independent pathways [5, 6]. During stabilization, α subunit dimerizes with the β subunit and then it binds with the hypoxia responsive elements of the promoter of target genes. In IVD, HIF-1α is the prime factor for maintenance of functional activities like proteoglycan matrix synthesis and also it is a critical factor for maintenance of nucleus pulposus cell survival and glycolytic metabolism [7-11].

Stem cells, which is present in various tissues is generally considered as having the ability to regenerate and differentiate into multiple lineages. Among the various types of stem cells, mesenchymal stem cells (MSCs) being a good source for different tissues. Also MSC derived from bone marrow or other tissues can easily be induced to form mesenchymal tissues, including fat, bone, cartilage [13] and non mesenchymal tissues such as neurons also can be induced to proliferate without differentiation [14]. Many previous *in vivo* studies have validated that MSC transplantation is promising for treating inter vertebral disc degeneration [15, 16]. However, the different culture environment and requirements for cell manufacturing, production and scale up are poorly understood. In *in vitro* culture condition different environmental factors like normoxic and hypoxic factors proved that promoting and altering the cell proliferation [17]. Many studies demonstrated that the hypoxic culture conditions enhance stemness and differentiation potential of MSCs *in vitro* into different lineages compared to MSCs cultured under normoxic culture conditions. Based on the normoxic and hypoxic culture, derived MSCs is referred as normoxic and hypoxic MSCs. Many studies have revealed that the hypoxia derived MSCs exhibited expanded migration to distant sites and it's engraftment after transplantation is more effective than normoxic culture derived MSCs [18]. Additionally, hypoxia MSCs produces more angiogenic cytokines, when compared with normoxic MSCs. Also recently it was proved that hypoxia MSCs has an increased ability to survive and engraft in allogeneic host tissues [19, 20].

Inflammatory responses in the central nervous and peripheral nervous systems play vital roles in the development and persistence of many types of pathological pain [21]. Certain inflammatory cytokines otherwise known as pro-inflammatory cytokines in spinal cord, cause many types of pain associated with the

generation of abnormal activity from compressed or injured nerve fibers or inflamed neurons [22]. Cytokines are small secreted proteins, otherwise known as immunoregulators released by cells have a specific effect on communications and interactions and also causes the inflammation. Based on the types of secreting cells, Cytokine is classified into different names like lymphokine (cytokines made by lymphocytes), monokine (cytokines made by monocytes), chemokine (cytokines with chemotactic activities) and interleukin (cytokines secreted by one leukocyte and acting towards other leukocytes). Also based on its actions on other cells, cytokines are divided into autocrine (act on the cells that secrete them), paracrine (on nearby cells) or endocrine (in some instances on distant cells). Mechanisms connected with disc degeneration and inflammatory cytokines remains elusive [22]. Generally reduced disc cell density is happening due to cytokine imbalance [23]. The effect of inflammatory response in intervertebral discs is determined by the balance between pro and anti inflammatory response of cytokines. Proinflammatory cytokines are predominantly produced and activated by macrophages, which are involved in the upregulation of inflammatory responses. In disc degeneration presence of interleukins like IL-1β, IL-6 and tumor necrosis factor TNF-α accelerates the process of degeneration [24, 25].

2. MATERIAL AND METHODS

2.1. Ethics statement

Here all the animals related to this experiment were carried out accordance with the guidelines for the care and use of animals for scientific purposes approved by the Institutional Animal Care and Use Committee. Six week old male mice BALB/c were used for cell preparation were obtained from TANUAS (Tamil Nadu Veterinary and Animal Sciences University). The mouse was euthanized by cervical dislocation under general anesthesia and the lumbar spine segments were dissected out under sterile condition using the Olympus SZX16 micro-dissection stereo microscope. The dissected and separated IVDs were rinsed with sterile PBS for few times and immediately placed in culture medium-containing 24-well cell culture plates and then it was used for further studies.

2.2. Isolation and culture of NPMSCs

According to the general procedure, the BALB/c mice euthanization was carried by cervical dislocation under general anesthesia induced by isoflurane inhalation and the disinfection of the skin was done with 70% ethanol to douse the mouse and to prevent the hair from spreading during dissection. Here the spinal column was removed from the surrounding tissues using scissors and the long bones, musculature, ribs and pelvis were removed to cut in a parallel direction to the visible spine from the sacrum and up to the thoracic region. Excised spinal column placed onto the iced dissection field under a dissecting microscope to isolate the IVDs from lumbar and thoracic regions. There was a smaller color change between the two structures. The isolated IVDs were placed on a small portion of the iced dissection field and the isolation of all other IVDs were carried out continuously. Observation of the isolated IVD, bone or soft tissue is visualized, these elements carefully removed by scalpel by holding the IVD and separating them away from the other tissues with a scalpel. With a 28 gauge needle, pierced the IVD from the superior to the inferior aspects, if NP is spared during dissection, it should be visible as a gelatinous out pouching, and placed in a previously prepared perforated tube within a tube, where a 0.5 mL micro centrifuge tube was punctured several times at its most inferior portion with a 25 guage needle and placed within a 1.5 mL micro centrifuge tube. Centrifuged at 12000 rcf for 3 minutes at 4°C. The NP cell mixture was collected in the 1.5ml micro centrifuge tube and the remaining AF/CEP found to be deposited in the bottom of 0.5mL micro centrifuge tube and then the cell mixture was transferred to a new 1.5 ml tube.

2.3. Culture of NPMSCs

NP tissues were removed from the mice by the above mentioned method as much as possible and then the tissues were digested with 0.2% collagenase II for 5-6 hours at 37°C . Then the cells were filtered with the help of a cell strainer containing the pore size of 70µm and resuspended in Dulbeccos modified eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The resuspended cells were seeded at a density of $1x10^5$ Cells/ml in 25 cm^2 flasks. Cells were cultured in DMEM incubated at 37°C in a humified atmosphere with 5% CO_2 till it reached 80% confluence. Then the medium was changed for every 3 days and the morphological changes of different types of NPMSCs was observed through inverted microscope and also passage three of NPMSCs cultures were used for other studies like proliferation and multipotent differentiation analysis. Also differentiation analysis of NPMSCs in normoxic and hypoxic culture setting, the normoxic

culture were carried out by placing the culture plates at 37°C with 5% CO_2 and the hypoxic culture condition was done with hypoxic chamber with 1%O_2, 5% CO_2 and 93%N_2.

2.4. Flowcytometry Analysis

NPMSCs phenotypic analysis was performed with Flowcytometry FC 500 (Beckman Coulter) according to the standards, which was proposed by International Society of Cellular Therapy (ISCT). Here the cell surface markers used for identification of NPMSCs phenotypes including CD11b, CD29, CD31, CD34, CD44, CD45, CD90, CD105, CD73, CD106, Sca-1, Ter -119 (Table.1) and HLA DR. Here the markers like CD34, CD45 and HLA DR were used as hematopoietic markers to differentiate the MSC markers and also those markers were highly expressed in hematopoietic stem cells. The other markers like CD29, CD44, CD73, CD90, CD105, CD106, Sca-1 were highly expressed in MSCs and also other markers like CD11b, CD31 and Ter -119 were used as negative markers and the MSCs lacked the expression of these markers. Here staining was done by the monoclonal antibodies related to phenotypic analysis, which was supplied by Beckman coulter (Beckman coulter, India). A total of $2x10^5$ NPMSCs cell from each culture was taken and suspended in PBS containing 1%BSA and 5% FBS to produce the single cell suspension for phenotypic marker analysis. First, the cells were stained with anti-mouse monoclonal (1:10 dilution) antibodies and then, it was incubated for 30 minutes at room temperature and cells were washed twice with PBS and finally it was diluted with 0.5-1ml of PBS for further analysis of surface marker expression by flowcytometery.

2.5. Cell Viability Assay

Here the Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-*2H*-tetrazolium bromide (MTT) assay kit (Abcam-USA, MTT Assay kit, ab21109) colorimetric assay. According to the standard methods described before (Wang et al., 2013), MTT is soluble in water (10 mg/ml), ethanol (20 mg/ml), and buffered salt solutions (5 mg/ml) and culture media. Cells were grown in DMEM media supplements with 10% FBS, Cells were harvested using trypsin and counted using Trypan blue dye exclusion assay with the help of hemocytometer. Serial dilution of Cells was done with a clear cell culture plate and incubated for 3 hours with MTT reagent at 37°C. Once incubation was complete, cells were treated with MTT solvent for 15 minutes at room temperature. After incubation at 37°C, 150 ml dimethyl sulfoxide (DMSO, Sigma) was added and the absorbance was measured at OD 590 nm with the help of a microplate reader.

2.6. Cell cycle and Apoptosis Assay

Cell cycle analysis from cultured NPMSCs was done by Flowcytometry from the passage three of NPMSCs and the cell was harvested from the cultured flask and it was washed twice with PBS containing 2% FBS. Then the cells were added to pre-chilled absolute ethanol and kept at 4°C for 1-2 hour to fix the cells. Then it was washed twice with equal amount of ethanol. The washed cells were added with 100µl of R Nase A and allowed to remain for 30 minutes at 37°C. The propidium Iodide (PI) (SIGMA, India) was added and kept at 4°C in the dark for 30 minutes and then the cell cycles were analysed by the CXP software. Quantification of apoptosis was performed by using an Annexin V:FITC Apoptosis Detection Kit (Abcam, Annexin V-FITC Kit ab14085, USA), according to the manufacturer's recommendations. Here 1-5 x 10^5 cells were collected by centrifugation. Here the Cells were resuspended in 500 µl of the diluted 1X Binding Buffer, Then 5 µl of Annexin V-FITC and 5 µl of propidium iodide (PI 50µg/ml, optional) was added. After 5 minutes of incubation at room temperature at dark condition, 300 ml phosphate buffer saline (PBS) was added into the cells and the apoptotic cells were distinguished under a flow cytometry (Beckman Coulter, India) immediately. Annexin V-FITC binding were analyzed by flow cytometry using signal detector FL1 and PI staining by the phycoerythrin (PE) emission signal detector FL2.

2.7. Sub culture of Mesenchymal Stem Cells

Media containing MSCs was removed and discarded from T75 tissue flasks and then the cells were washed with PBS. To remove adherent cells 1-2 ml of pre warmed trypsin were added into the T75 flask containing MSCs and it was incubated at 37°C for 1 minute. The dissociated MSCs transferred into the 15ml conical tubes afterwards, it was centrifuged for 5 minutes at 400xg. Then the cell pellets were resuspended in the 5 ml of stem XVIVO expansion media and then the cells were counted. $3.5\text{-}4.0x10^5$ MSCs was added and resuspended in 20ml of pre-warmed stem xvivo MSC expansion media to each T 75 flasks.

2.8. Multipotent Differentiation

Multipotent differentiation analysis of NPMSCs from different culture groups were used for differentiation of Adipogenic, Osteogenic and Chondrogenic lineages. All the procedures were done in accordance with the guidance provided by the manufactures of the differentiation medium.

2.9. Osteogenic Differentiation

Osteogenic differentiation was noted in normoxic and hypoxic culture condition. Passage 3 of NPMSCs was seeded in 6 well plates containing osteogenic differentiation medium (Stem cell technologies) at the density of 4 - 6 x 10^4 ml. Here the cells were plated in appropriate culture medium (*e.g.* complete Mesen Cult™ Expansion Medium with or without MesenPure™) and culture condition, for normoxic culture cells was cultured at 37°C in a humified atmosphere with 5% CO_2, For hypoxic culture Cells were incubated at 37°C under hypoxic condition until it reaches approximately 80-90% confluent. Generally it takes 3 days. Then the medium was aspirated and replaced with new complete osteogenic medium. Cells were incubated at 37°C in hypoxic condition as well as the medium was changed for every 3 days until the bone matrix formation was observed. This took approximately 14-21days. Osteogenic differentiation was detected by Alizarin Red S staining and observed under the inverted microscope and its specific transcripts were analyzed through qPCR.

2.10. Adipogenic Differentiation

NPMSCs cells were plated in appropriate culture medium (*i.e.* stem cell technologies complete Mesen Cult™ Expansion Medium with or without Mesen Pure™). For normoxic, cells were cultured at 37°C in a humified atmosphere with 5% CO_2, For hypoxic condition (5% O_2 and 5% CO_2) until it reached approximately 80-90% confluent. Generally it takes 3 days. Then the medium was aspirated and replaced with new complete osteogenic medium. Cells were incubated at 37°C in hypoxic condition and the medium was changed for every 3 days with adipogenic differentiation medium until the lipid droplets were observed. Adipogenic differentiation was detected by Oil Red O staining and its specific transcripts were analyzed through qPCR.

2.11. Chondrogenic Differentiation

NPMSCs were resuspended in 2 ml of chondrogenic differentiation medium in the concentration of 2x10^6 cells at room temperature (15-25°C). 0.5ml of cell suspension was added into the 4x15 ml of the polypropylene tubes and centrifuged at 300xg for 5-10 minutes at room temperature (15-25°C). Cells were incubated at 37°C and 5% CO_2 for 3 days. On the day 3, 0.5ml of chondrogenic differentiation medium was added to each tube at room temperature (15-25°C) till it reached the final volume of 1 ml. On the day 6 and every three days afterwards the medium was aspirated without disturbing the pellet. The tubes were replaced with 0.5 ml of chondrogenic differentiation medium at room temperature (15-25°C). The tubes were incubated at 37°C in the presence of 5% CO_2. After the every medium change, it was ensured that the pellet was not completely attached to the tube by gentle flick and the pellet size gradually increased throughout the incubation period. On the day 21, the chondrogenic pellets were found to have full differentiation and further it was used for phenotype characterization by flowcytometry next to that it was confirmed by qPCR analysis. After 28 days of cell culture, finally the cells were fixed with 10% formalin at room temperature (15-25°C) for 30 minutes, followed by subsequent standard paraffin embedding methods and stained with 6µm sections of Alcian blue (Sigma Aldrich, india).

2.12. Cell proliferation Assay

NPMSCs from passage three were selected from six well seeded plates and cultured with DMEM supplemented with 10% FBS. Cells were derived from 14 days of culture and then the cells were fixed with 4% paraformaldehyde for 15 minutes afterwards, it was stained with crystal violet. Cells were washed with distilled water up to three times and colonies having >30 nucleated cells were used for counting with an inverted microscope.

2.13. Colony Formation and cytotoxicity Assay

100µL of cell suspension (5000 cells/well) was dispensed in a 96-well plate. Plates were pre-incubated for 24 hours in a humidified incubator at 37°C, 5% CO_2. 10µl of various concentrations of the substance to be tested were added to the plate. Plates were incubated for an appropriate length of time (6, 12, 24, 48 hours and 1, 3, 5, 7, 9, 11 and 13 days in the incubator. 10µl of CCK-6 solutions were added to the each well of the plate without bubbles. Incubation of the culture plates was done for 1-4 hours in the incubator. OD absorbance was measured at 450nm by using a microplate reader.

All experiments were performed three times in triplicate at each time point.

2.14. Real Time PCR(RT-PCR) gene expression analysis

Gene expression analysis of NPMSCs was carried out through the mRNA analysis by real time PCR. NPMSCs were expanded up to passage 5 and were characterized by stemness gene expression and multiple differentiation. The expression levels of stemness genes (Nanog, Sox2, Rex-1 and Oct-4) and The expression of hypoxia-inducible factor (HIF)-1α along with its associated genes like transporter (GLUT)-1, GLUT-3 and collagen II, aggrecan, matrix metalloproteinase (MMP)-13 and tissue inhibitor of MMPs (TIMP)-1 was assessed from the cultured NPMSCs (Table 2). Also an expression of pro-inflammatory cytokines IL-1, IL-6 and TNF-α was assessed. The total RNA was isolated from the cultured NPMSCs using TRIzol (Invitrogen) following the manufacturer's instructions. Here the c-DNA is formed from the six hundred nanograms of extracted mRNA and then it was reverse transcribed by using 1µl of oligo (dT) primer (Invitrogen), 1 µl of dNTPs and 200 U of reverse transcriptase (Superscript II, Gibco) at 42°C for 60 minutes. Then the reaction stopped by incubating the reaction tube at 95°C for 5 min. The quantification of m RNA was carried out with Qiagen rotor gene 6000 in the following temperature profile and amplified for 45 cycles at 95°C/10s, 62°C/25s with the specific primers. Here the Ct values were used for the interpretation of gene expression analysis in both hypoxic and normoxic cultures derived NPMSCs association with inflammatory cytokines.

3. RESULTS

3.1. NPMSCs Characterization

Characterization of NPMSCs was done through the surface markers and gene expression analysis by Flowcytometry and real time PCR. Here the cells expressed the mesenchymal stem cells (fig. 2a) such as CD29, CD44, CD90, CD105, CD73, CD106 and Sca-1 and lacked the expression of hematopoietic markers, those are CD34, CD45 and HLA DR (fig. 2b). Also NPMSCs were characterized by differentiation into adipogenic, Osteogenic and chondrogenic culture and confirmed with staining (fig.1). Adipogenic differentiation was observed through the formation of lipid droplets through Oil Red O staining from the cultured NPMSCs after 14 days of culture in adipogenic differentiation medium. Also adipogenic differentiation observed through subsequent qPCR analysis of specific transcripts like FABP4 (fatty acid binding protein 4) and PPARs (peroxisome proliferator activated receptor γ). Osteogenic differentiation was observed through bone matrix formation from the cultured NPMSCs in osteogenic differentiation medium and it was confirmed with the staining of Alizarin Red and visualized mineralization and further analysis were carried out by qPCR analysis of bone specific transcripts Runx2 and Osteocalcin. For chondrogenic differentiation cells were stained with Safranin O and it's specific characteristic genes like sox 9 and col II analyzed through q PCR.

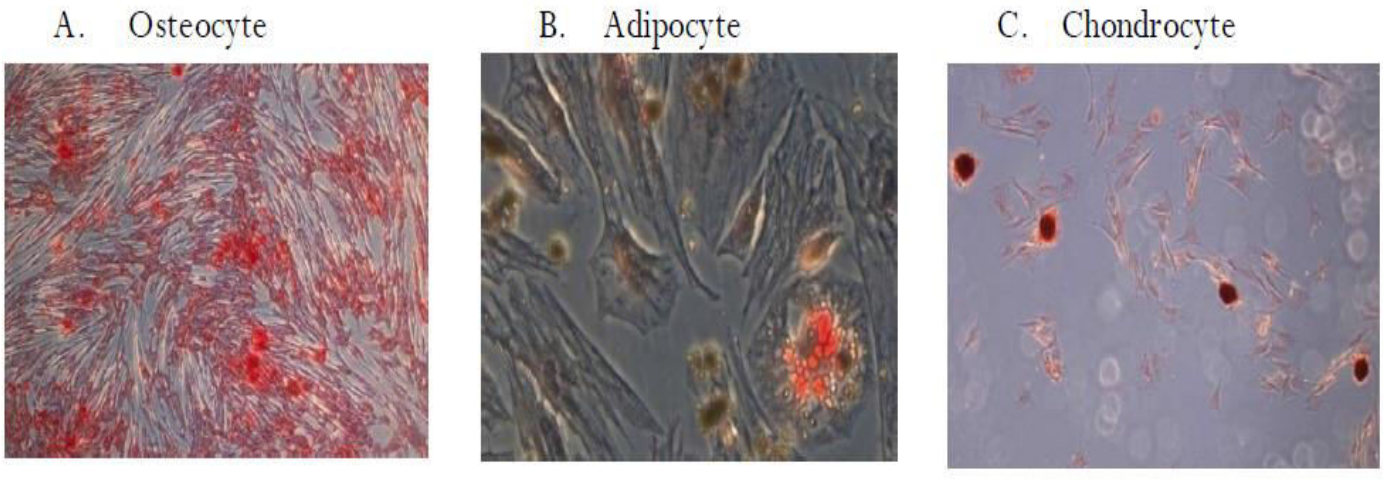

Fig. 1: Immunohistochemistry analysis of NPMSCs multi differentiation into osteogenic, adipogenic, chondrogenic and its confirmation through alizarin red staining for osteocytes, oil red staining for adipocytes and safranin O staining for chondrocytes

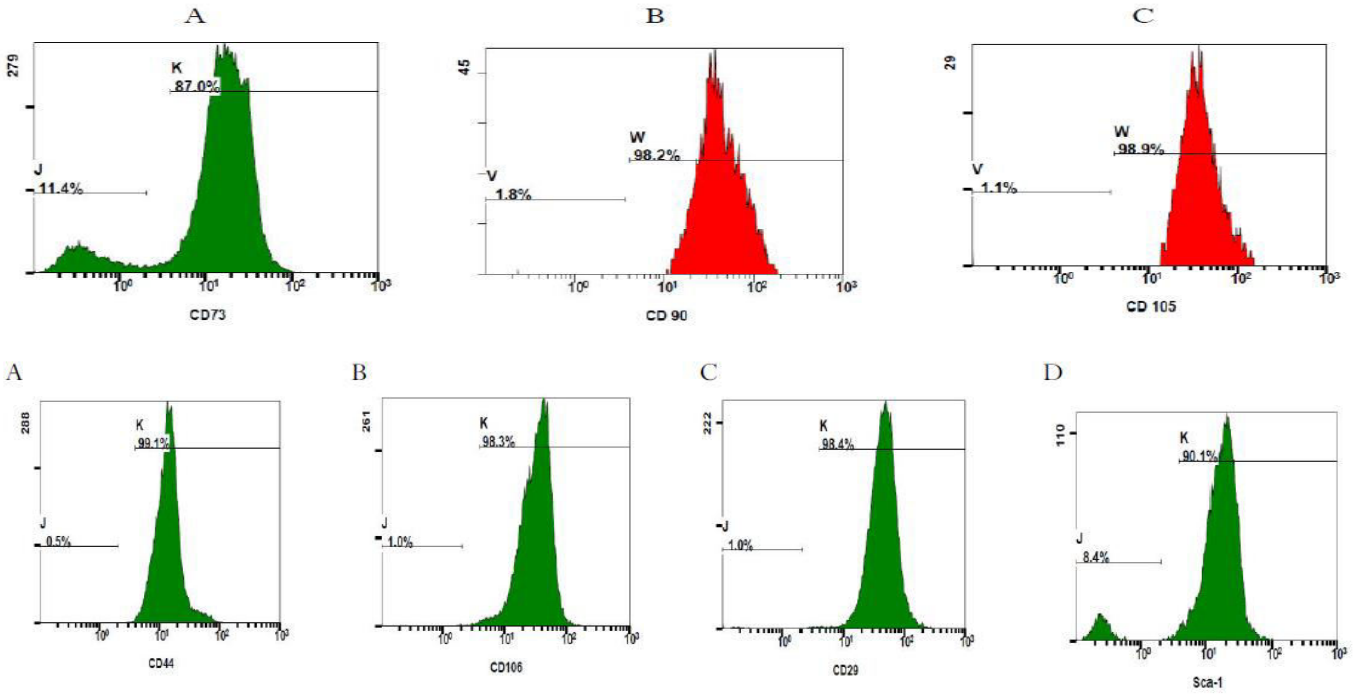

Fig. 2a: Nucles pulposus mesenchymal stem cells (NPMSCs) identification. Flowcytomerty analysis of surface markers CD73, CD90, CD105,CD44 , CD106 , CD29, Sca-1 showed high expression

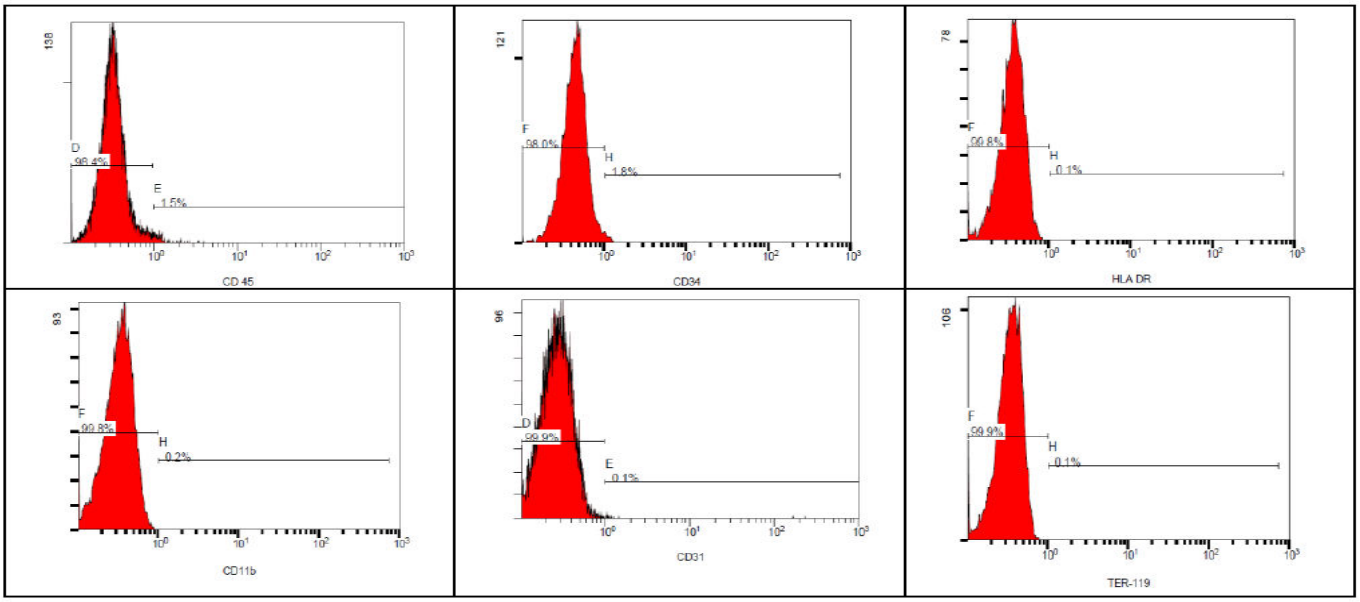

Fig. 2b: Flowcytomerty analysis of hematopoietic stem cells surface markers CD45, CD34, HLA DR and NPMSCs negative selection surface markers CD11b, CD31 and Ter-119 showed low expression in negative region of histogram

3.2. Inflammatory Cytokines increase the cell death

To measure the cell proliferation of NPMSCs in normoxic and hypoxic culture environment in the presence of the inflammatory cytokine, cell cycle and apoptosis was performed by Flowcytometry (fig. 5). In the absence of pro-inflammatory cytokines in normoxic as well as in hypoxia culture setting level of the apoptotic cells was normal. The experiment revealed that in the presence of inflammatory cytokines the NPMSCs in both normoxic and hypoxic culture condition shows higher ratio of apoptotic cells, when

compared with the cytokines free culture. Alternatively in the presence of inflammatory cytokines, the presence of dead cells was greatly increased in normoxic as well as in hypoxia culture condition.

3.3. Influence of normoxic and hypoxic environment on NPMSCs

Proliferation of cultured NPMSCs was noted in normoxic and hypoxic culture setting and then it was found that Hypoxia keep up the viability of NPMSCs and the numbers of viable, apoptotic and secondary necrotic cells were determined by annexin-V-FITC/PI double staining. Here the proliferation of NPMSCs was significantly influenced by the oxygen concentration and an interaction between oxygen concentration and NPMSCs. Proliferation of NPMSCs was monitored on days 7 and 14. The proliferation of NPMSCs on the day 7 of the culture condition was normal and it was significantly higher under hypoxia, when compared to normoxia. Also similar results were observed on day 14 as the proliferation was even better under hypoxia when compared with normoxia (fig. 5). Here the phenotypic expression of NPMSCs was monitored in both culture environments. In the presence of hypoxic culture condition NPMSCs greatly upregulated the expression of phenotypic markers like CD44, CD166 and CD105 when compare with the normoxic culture condition.

3.4. Hypoxic Responsive genes Expression

The expression of hypoxia responsive genes like HIF-1α, GLUT-1, GLUT-3 and VEGF-A from the cultured NPMSCs were found to be expressed in an upregulated manner. The results indicated that there was a significant influence of oxygen concentration on cell types. Gel electrophoresis was carried out to confirm the presence of these genes expressions (fig. 3a) and its further quantitative analysis of these genes performed through realtime PCR. Ct value was calculated for all the genes to find its expression level in a different culture setting. Expression of these genes was monitored on day 0, 7, 14 and 21 in both culture background. The overall percentage of HIF-1α in from normoxic culture derived mRNA was 29 and in hypoxia was 24 (fig. 4), For GLUT-1 the normoxic culture derived Ct was 31 and in addition to that the hypoxic culture derived Ct was 27. At the same time the GLUT-3 Ct value in normoxic found to be 30 and in hypoxic it was 27. Following to that, the Ct value of VEGF-A in normoxic 30 and hypoxia was 26. Here the Ct value is inversely proportional to the gene expression. Interaction between oxygen concentration and cell types greatly varied these gene expressions. The significant influence of oxygen concentration and an interaction between oxygen concentration and cell types for HIF-1α, GLUT-1, GLUT-3 and VEGF-A, found to be altered. Quantitatively the expression levels of those genes were less in NPMSCs under normoxia. In addition to that, when it compares with normoxia, the expression levels of HIF-1α, GLUT-1, GLUT-3 and VEGF-A were greatly higher in NPMSCs under hypoxia.

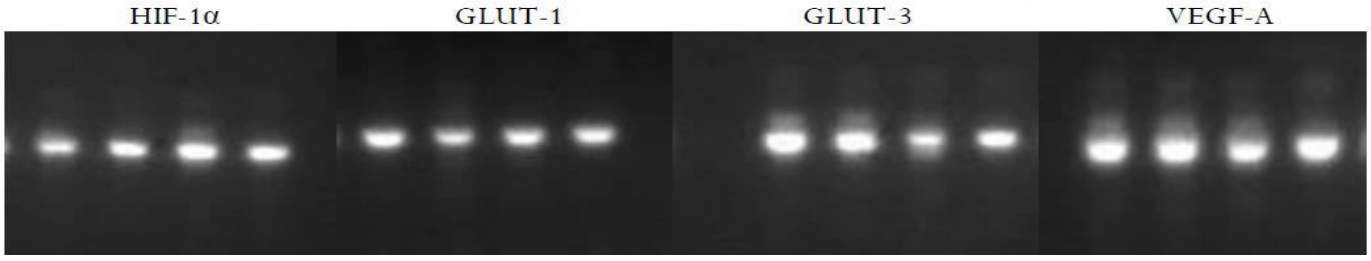

Fig. 3a: Normoxic Culture derived genes expression

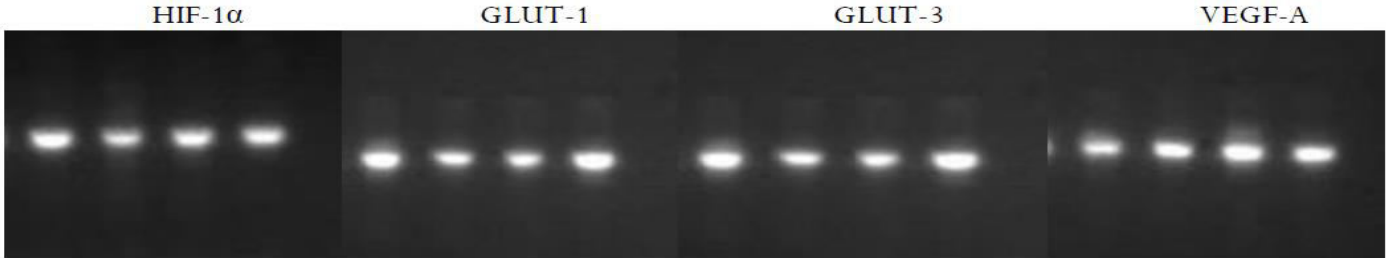

Fig. 3b: Hypoxic Culture derived genes expression

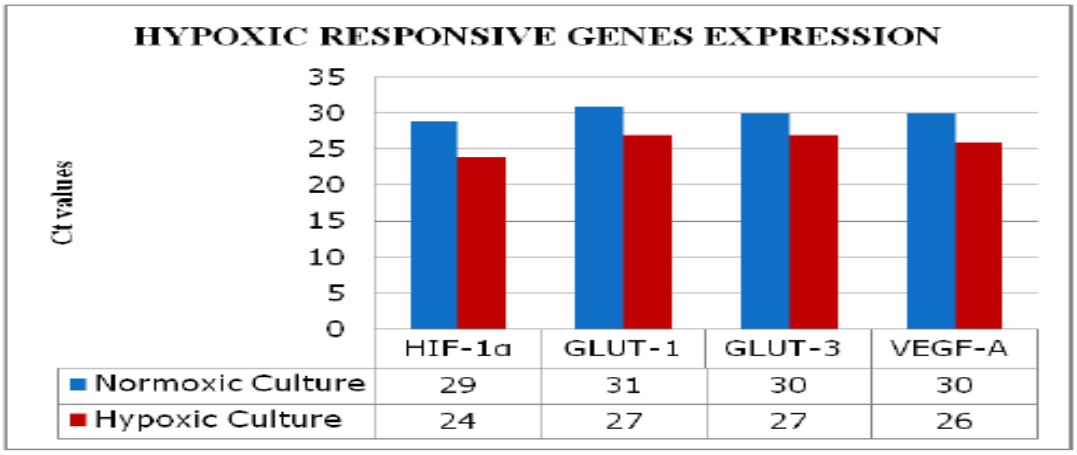

Fig. 4: Differential expression of genes like HIF-1α,GLUT-1,GLUT-3 and VEGF-A in normoxic and hypoxic culture setting analysed through realtime PCR and its Ct value reveals the genes significantly expressed high in hypoxic culture setting, while it compares with the normoxic culture derived genes. Here the Ct value is inversely proportional to the gene expression

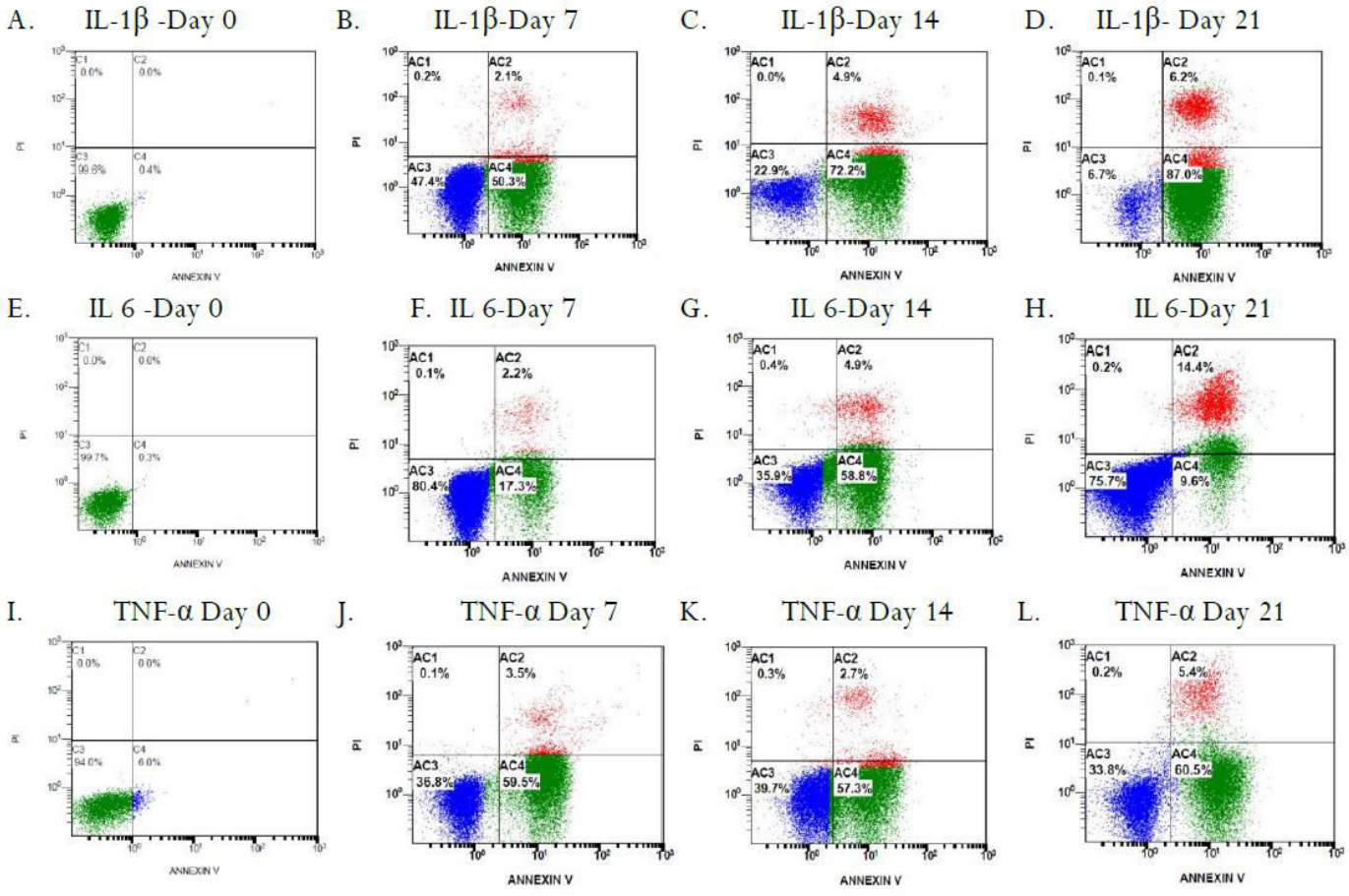

Fig. 5: Flowcyometric analysis of cell apoptosis rate of NPMSCs treated with inflammatory cytokines like IL-1β(A-D), IL-6(E-H) and TNF-α(I-L) on Day 0,7,14 and 21 and stained with Annexin V and Propidium Iodide (PI) shows the normal apoptotic rate on day 0, But in the presence of pro-inflammatory cytokines on Day 0, 7, 14 and 21 exhibits increased amount of cell death in each consecutive passages while compare with on day 0 culture.

3.5. Influence of Inflammatory cytokines in hypoxic culture condition

Pro-inflammatory cytokines like IL-β, IL-6 and TNF-α were added in the culture plate containing the NPMSCs on day 0, 7, 14 and 21 of different culture groups. In general, NPMSCs exhibits great proliferation potential at hypoxic environment, it's proliferation efficiency, monitored in the presence of Pro-inflammatory cytokines at day 0, 7, 14 and 21. In this assay, the Pro-inflammatory cytokines was added individually in hypoxic culture setting. The viability of NPMSCs was noticed in hypoxic culture condition. The results showed that there was a significant influence of Pro-inflammatory cytokines on the hypoxic environment (fig.5). All the three cytokines in hypoxic culture greatly reduces the expression of hypoxia resistant genes, when comparing with the day 0 of culture group. From these studies, it was revealed that the Pro-inflammatory cytokines effectively inhibits NPMSCs proliferation on successive passages and increase the number of apoptotic and dead cells of NPMSCs on each consecutive passages.

4. DISCUSSION

Disc disorders are mainly preceded through pain by the changes, first occurring in an NP region of the discs. It is characterized by changes in cell function, extracellular matrix composition, tissue cellularity and morphological distinction [26, 27]. Subsequently, the regeneration of the degenerated discs is very limited due to the cellularity decline and phenotypic changes. Mesenchymal Stem Cells from various adults have been tested for regeneration of disc degenerative tissues for many years to treat DDD [28-32]. Also, several studies have been attempted to use the Nucleus pulposus derived mesenchymal stem as a source for cell therapy for inter vertebral disc degeneration. [33-36]. For many years, NPMSCs collected from nucleus pulposus is being used for IDD therapeutics and also the isolation and culture of nucleus pulposus cells is being a cumbersome process due to it's low cell activity [37]. The aim of present study was to evaluate the characterisation and biomarkers analysis of normal and degenerated nucleus pulposus containing discs derived mesenchymal stem cells along with how it's regeneration and differentiation characteristics affected by pro-inflammatory cytokines, when it is subjected to the different characteristics like normoxic and hypoxic culture condition. To prove this, we isolated the normal and degenerated nucleus pulposus from the mice and successfully cultured as well as it's genotypic and phenotypic characters were analyzed. Here both degenerative and non degenerative NP derived mesenchymal stem cells expressed the similar positive phenotypic markers like CD29, CD44, CD90, CD105, CD73 and CD106. Also both mesenchymal stem cells expressed the similar multi differentiation potential.

In general the NP cells are considered as producer of the molecular components of the ECM to maintain the structural integrity of the IVD. In addition to that, numerous studies demonstrated that apoptosis decrease the efficiency of NP cells and make changes in the mechanism of IVD development [38, 39]. Hitherto, several studies revealed that inflammatory cytokines are prime regulators of the apoptosis of NP cells [40]. Most of the studies proved that, when NP cells undergo the inflammatory cytokines like IL-6, IL-1β and TNF-α treatment, It exhibits the phenotypic variation [40]. Here the foremost object of this work was to find the interrelation of pro-inflammatory cytokines and hypoxic inducible factor. In this study, we used both normal and degenerated discs derived NPMSCs, but for the most of the studies we utilized the normal NP tissues derived cells in normoxic and hypoxic culture condition. Overall, the NPMSCs is having well differentiation potential at hypoxic environment, But here we noted, it's differentiation potential in both culture conditions. In normoxic culture condition, both NPMSCs showed almost similar differentiation potential. In the same time, degenerated NPMSCs cells shows the much senescent cells. In the presence of inflammatory cytokines we compared the effect of inflammatory cytokines for both normal and degenerated NPMSCs in normoxic and hypoxic culture condition. In normoxic culture condition NPMSCs shows the normal proliferation capacity, but in the presence of inflammatory cytokines, it shows a less proliferation capacity in the same normoxic culture condition. Likewise, we analyzed the proliferation level of the NPMSCs in hypoxic culture condition. Generally NPMSCs shows expanded proliferation level in hypoxic culture setting, but here when we subjected that NPMSCs in hypoxic culture, it showed the good proliferation same time in addition of inflammatory cytokines it exhibits reduced proliferation. From these it was revealed that inflammatory cytokines deter the proliferation and differentiation potential of NPMSCs in both normal and hypoxic culture environments. Also genotypic analysis of NPMSCs analyzed from both normal and hypoxic culture in the presence of inflammatory cytokines. We

compared HIF1-α, GLUT-1, GLUT-3, VEGF-A, MMP-3 and MMP-13 gene expression of NPMSCs in both culture conditions. In hypoxic culture, it's expression was high when compared with normoxic culture, parallelly in the addition of inflammatory cytokines it's expression was greatly reduced in hypoxia as well as normoxic culture. Hence, it was proved that these pro-inflammatory cytokines reduce the proliferation of NPMSCs at both culture conditions.

5. CONCLUSION

In conclusion, the present study demonstrated that the NPMSCs from both normal and degenerated tissues expressed the similar phenotype and genotypes. All the differentiation characteristics like cell proliferation, stemness gene expression, cell migration and self renewal capacity was higher in normal tissue derived NPMSCs, when compared with degenerated tissue derived NPMSCs. The study mainly focussed on growth of NPMSCs in different culture setting along with the effect of pro-inflammatory cytokines on that culture. Also NPMSCs shows better proliferation in normoxic and hypoxic culture environment. Though, the NPMSCs showed the good proliferation in both types of culture condition, it exhibits higher rates of proliferation in hypoxic culture condition. But in the presence of inflammatory culture condition it was demonstrated that its proliferation was deterred. Hence the effect of inflammatory cytokines was noted in both types of culture conditions. In such case, it was found that inflammatory cytokines increased the caspase gene expression activities and also it prevents the proliferation of NPMSCs in both normoxic and hypoxic culture conditions. Also inflammatory cytokines down regulate the expression of hypoxia genes like HIF-1α, GLUT-1, GLUT-3 and VEGF-A. It was found that in the presence of inflammatory cytokines addition of the anti inflammatory cytokines like IL-10 in both culture environments promote the cell proliferation and gene expression.

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Conflict of Interest

None Declared

6. REFERENCES

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