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IN-VITRO CULTURE CHARACTERISTICS OF YOUNG AND OLD RAT MESENCHYMAL STEM CELLS

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Keywords:

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ABSTRACT: Mesenchymal stem cells are a small number of undifferentiated cells located in various tissues of the body. They have the ability to self-renew and differentiate into lineages of mesenchymal origin. They serve as most promising candidates for various regenerative medicine and tissue engineering applications. Their use in various clinical applications depend on their proliferative capacity to expand cell population. One of the key challenge to expansion is decline in proliferation and differentiation capacity of cells isolated from older individuals. In the present study, we have compared cellular characteristics of bone marrow-derived mesenchymal stem cells isolated from young (4 – 6 weeks age) and old (60-64 weeks age) rats. The two rat groups were compared for proliferation kinetics, differentiation potential and senescence markers SA- βgal, reactive oxygen species (ROS), as well as expression of$P16^{INK4A}$, P21 and telomerase genes. A significant decrease in frequency, population doubling time, and cell yield associated with increase in age were observed. A decline in differentiation potential for osteogenic and chondrogenic lineages, and an enhancement in adipogenic lineage correlating with increase in age were noted. A significant proportion of old MSCs was SA β- galactosidase positive and had enhanced levels of ROS. The gene expression studies showed an up-regulation of senescence specific $p16^{INK4a}$, p21 genes and down-regulation of telomerase gene in aged cells. Our findings determine the culture characteristics of old and senescent MSCs. It may be of value in developing strategies for retrieval of optimum functioning cells among the population undergoing cellular senescence.

INTRODUCTION: Mesenchymal stem cells (MSCs) are non-hematopoietic cells residing in bone marrow, adipose tissue, skeletal muscle, spleen, synovial membrane, and other body tissues. They are present in very small numbers and comprise less than 0.0001 percent of the total number of cells.

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They remain quiescent and possess the capacity for self-renewal and differentiation after an injury, dysfunction, or aging. An adequate stimulus may mobilize and release MSCs to migrate to injured sites for the regeneration process [1].

Regeneration of tissue is a complex phenomenon and involves the integration of the regenerated cells with the surrounding tissues and their differentiation through the natural signaling pathways [2]. MSCs can migrate to the specific site of injury and remain stable for a long period. They differentiate into a variety of cell lineages, including osteoblasts, myoblasts, adipocytes,

tenocytes and even neurons [3]. They secrete a spectrum of growth factors, cytokines and chemokines, including IL-1a, IL-1b, IL– 6, IL-7, IL-8, IL-11, IL-14, IL-15, macrophage colony-stimulating growth factor, granulocyte-macrophage colony-stimulating growth factor (GM - SCF), leukemia inhibitory factor, stem cell factor which play a key role in tissue regeneration process. MSCs have ability to modulate immune response. They can inhibit alloreactive T lymphocytes. They do not express MHC Class II molecules or co-stimulatory receptors on their surface and hence can escape antigen-presenting cell-mediated response. They also exert immunosuppression by inhibiting T cell responses to polyclonal stimuli and to their cognate peptide. The inhibition is caused by arresting T cell proliferation in G1 phase. MSCs also have an effect on B cells and dendritic cells to modulate the immune system [4].

The self-renewal, multipotent differentiation, homing, tissue regeneration, and immuno-modulatory properties of MSCs make them the most preferred choice of cells for regenerative and tissue engineering applications. Cellular therapies such as given in osteogenesis imperfecta, graft versus host disease, bone/cartilage lesions, myocardial infraction, neuronal lesions, and spinal cord injury underline the potential of MSCs in the treatment of various non-curable diseases [5].

Among the various sources of MSCs, those isolated from the autologous source are preferred for most therapeutic applications. An adequate number of cells presenting similar immune-modulatory, homing and differentiation properties are key requirements of these cells for successful cellular therapy. Retaining a stable phenotype of cells presenting these properties is however a major challenge faced during MSC *in-vitro* propagation. Typically, they tend to undergo cellular senescence after few passages *in-vitro* or after a certain age *in-vivo*. They show a reduction in proliferation and differentiation potential. An elevated expression of the p53 gene and its pathway genes, p21, p16NKb, has been detected in cells isolated from aged individuals [6]. These proteins are involved in accelerating senescence and degrading of different cells and tissues such as fibroblasts, endothelial cells, myocardium, and bone. The generation of higher volumes of reactive oxygen species and nitric oxide have also been noted in this process. A combination of all these factors contributes to compromised activity and age-related loss of MSC performance [7]. The senescence potentially deteriorates MSC characteristics to the point where the residual stem cells are unable to support tissue regeneration.

The present study was undertaken to correlate the cellular characteristics with the age of MSCs. The mesenchymal stem cells isolated from 4 to 6 weeks young and 60 to 64 weeks old rats were compared for cell proliferation, differentiation and senescence parameters. They were evaluated for proliferation kinetics, differentiation potential, SA β-galactosidase staining, generation of reactive oxygen species and expression of senescence genes telomerase, P $16^{INK\ 4a}$ and P21. The study was aimed at comparing the culture characteristics of MSCs isolated from young and old age groups. It is worthwhile to know the properties of cells undergoing cellular senescence in order to select the appropriate cell population for clinical applications.

MATERIALS AND METHODS:

Isolation and Culture of Rat Bone Marrow Mesenchymal Stem Cells: Use of rats for present study was approved by Institutional Animal Ethics Committee (Ref no: BVDUMC/3305/2016/019/ 002) and the procedure followed was in accordance with CPCSEA guidelines. The primary culture preparation was performed using the procedure described by Zhang *et al* [8]. Briefly, a Sprague dowley rat of young (4-6 weeks old) and old (60-64 weeks old) age were anesthetized using diethyl ether and dissected. Tibia and femur bones were isolated. The bone marrow was flushed and collected in centrifuge tubes containing growth medium (α-MEM supplemented with 10% MSC qualified serum, 50 U/ml penicillin, and 50µg/ml streptomycin).

Pellet was collected after centrifuging the cell suspension at 2000rpm for 10 mins. Pellet was washed twice with PBS and resuspended in a growth medium. The cells were seeded in 6 well plates and incubated at 37 ºC in 5% CO_2 atmosphere. Regular media change was given after every third day. The cultures after attaining confluency were subcultured, and cells from third passage were used for subsequent experiments.

Growth Curve Assay and Determination of Population Doubling Time (PDT): About 1×10^4 cells were seeded in each well of 24 well plates and incubated at 37 ºC in 5% CO_2 atmosphere. The cells from duplicate wells were trypsinized after every 24 hours interval and counted using trypan blue dye till cells reached to confluency (7days for YR and 15 days for OR MSCs, respectively). Population doubling time was calculated by using the following formula.

$$PDT = CT/PDN$$

$$PDN = \log (N/No)\times3.31$$

Where,
N- Number of cells at the end of growth period
No- Initial number of cells
CT- Time of cultivation between passages
PDN- Population Doubling Number

Adipogenic Differentiation: About 1×10^5 cells were seeded in 24 well plates and incubated at 37 ºC in 5% CO_2 atmosphere for 24.00 hrs. The cells were then switched to an adipogenic induction medium (Gibco by Life Technologies) and further incubated for 7 days. Media change was given after every 3 days. The cells were then fixed in 4% paraformaldehyde, washed and stained with 0.3% Oil Red-O solution. The cells were observed and photographed using ZEISS Axiocam Microscope Camera. Oil Red O was eluted by adding 100% isopropanol and followed by incubation for 3.0 hrs. Absorbance was measured at 500 nm using 100% isopropanol as a blank. The absorbance of young and old rat age group were plotted and compared.

Chondrogenic Differentiation: 1×10^5 cells of third passage MSC were seeded in 24 well plates and incubated at 37 ºC in 5% CO_2 atmosphere for 24.00 h. The cells were switched to chondrogenic induction medium (Gibco by Life Technologies) and further incubated for 14 days. Media change was given after every 3 days. After 14 days, the cells were fixed with 4% paraformaldehyde, washed and stained with 1% Alcian Blue dye. Quantification of the aggrecans formed was performed by eluting Alcian Blue dye by adding 8M Guanidine HCL solution and incubating over night at 4 °C. Optical density was read at 600nm using 8M Guanidine HCl as blank. The absorbance versus rat age group were plotted and compared.

Osteogenic Differentiation: 1×10^5 cells of passage three BM-MSC cultures were seeded in 24 well plates and incubated at 37 ºC in 5% CO_2 atmosphere for 24.00 hrs. The cells were then incubated in an osteocyte differentiation medium (Gibco by Life Technologies) for 21 days. Media change was given after every 3 days. The cells were then fixed using 4% paraformaldehyde, washed, and stained with 2% alizarin red dye. The dye was eluted with 10% acetic acid followed by three freeze and thaw cycles. Lastly, 10% Ammonium hydroxide was added to neutralize acid. The absorbance was read at 405nm and plotted against the rat age groups.

SA-β-Galactosidase staining: Senescence-associated β-galactosidase (SA-β-gal) staining was performed using EZdetect™ (HiMedia Cell Culture, CCK 063) cell senescence detection kit. The procedure followed was in accordance with the kit instructions. In brief, 1×10^5 MSCs were seeded in 24 well plates and incubated for 48.00 h at 37ºC in 5% CO_2 atmosphere. After incubation, cells were fixed with 4% paraformaldehyde for 5 min. The cells were washed and stained with SA-β-galactosidase–staining solution and incubated for next 12.00 hrs at 37 ºC without CO_2 atmosphere. The cells were then washed with PBS and observed. The SA-β-gal–positive cells appeared in blue color. The number of SA-β-gal–positive cells were counted and photographed using ZEISS Axiocam Microscope Camera. The percent senescence cells of the two rat age groups were compared.

ROS Staining: The cellular reactive oxygen species were detected by using DCFDA cellular ROS detection assay kit (Abcam, ab113851). The 2', 7' -dichlorofluorescindiacetate (H2DCFDA) fluorescence intensity of the cells was detected by flow cytometry. Briefly, 1.5×10^5 cells were collected in a conical test tube and washed with 1X assay buffer provided with the kit. The cells were stained with 20µM DCFDA for 30 min at 37 ºC. Cells were washed once with 1X buffer. Signals were immediately detected using FACS scan flow cytometer (BD Bioscience) with excitation at 485 nm and emission at 535nm.

Gene Expression: The expression of senescence specific genes telomerase, p21, $p16^{INK4A}$ was

evaluated using quantitative real-time PCR. Total RNA was isolated using TRIzol reagent (In-vitrogen), and c-DNA was synthesized using an oligo (dT) primer and superscript III reverse transcriptase enzyme (In-vitrogen). Power up SYBR green master mix kit. (Applied Bioscience) was used for real time PCR experiments. The procedure was conducted according to manufacturer's proto-col. The changes in gene expressions were evaluated by normalizing the PCR signal to that of GAPDH (Glyceraldehyde-3-phosphate dehydro-genase). Each reaction was run in triplicate and the entire procedure was repeated three times.

Statistical Analysis: The experiments were performed in triplicates. The data presented is mean of replicates. Significance was determined using paired t-test. P-valueless than 0.05 was considered for statistical significance. *, ** and *** in the graphs indicate significance < 0.05, < 0.01 and < 0.001 respectively.

RESULTS:

Primary Culture of Rat Bone Marrow Mesenchymal Stem Cells: The *in vitro* cultures of MSC isolated from the bone marrow of young (4 to 6 week's age, YRMSC) and old (60-64 weeks age, ORMSC) rats were compared for growth characteristics, differentiation potential and senescence parameters in the present study. **Fig. 1** shows the microscopic images of cell morphology and cell density exhibited by YRMSC **Fig. 1A** and ORMSC **Fig. 1B** on days 5, 10, and 15 in primary culture. The YRMSC adhered to the substrate in 48 to 72 h and entered the log phase.

They exhibited typical fibroblastic spindle-shaped morphology. They multiplied readily in the culture and attained confluency in the span of 17 to 19 days. The cells were much crowded at confluency and displayed an average cell count of 6.7×10^5 cells in a single well of a six-well plate.

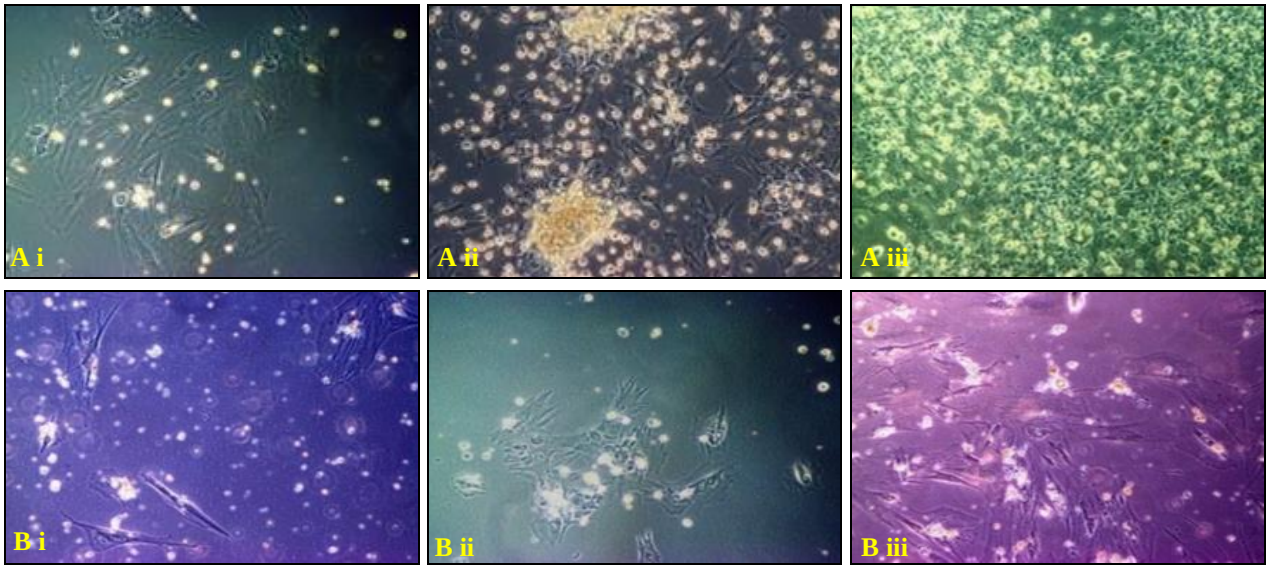


FIG. 1: MICROSCOPIC IMAGES SHOWING MORPHOLOGY AND CELL NUMBER OF MESENCHYMAL STEM CELLS DERIVED FROM YOUNG RAT AND OLD RAT BONE MARROW. A (i - iii), and B (i - iii) show the microscopic images of young and old rat MSCs captured on days 5, 10, and 15 of primary culture, respectively.

The cells of ORMSCs adhered to the plate and showed a lag phase of about 7 days. They proliferated thereafter but exhibited much enlarged and flattened morphology. They were less associated with each other. The rate of multiplication was slower and reached to confluency stage after 26 to 28 days. The average cell count of old rat cells was 3.3×10^5 cells per well of six well plate showing much-reduced cell yield as compared to the young cells. The cells were subcultured at this stage, and new cultures were seeded at a cell density of $1x10^5$ cells. The rate of cell proliferation enhanced in subsequent passages compared to primary culture. The duration for passaging of young rat MSC was 7 – 9 days, whereas for old rat, it was 15 - 17 days. The growth characteristics of the young and old cells were compared using a growth curve assay.

Growth Curve Assay: Equal number of cells (1×10^4) of the third passage MSC culture were seeded in each well of 24 well plates. Plates were

incubated in 5 % CO_2 atmosphere. Cells from duplicate wells were trypsinized and counted using trypan blue dye after every 24 h until the cells reached confluency. The cell count was plotted against time. The graphs are presented in **Fig. 2A** and **B**. The rate of cell proliferation was estimated using the formula described earlier.

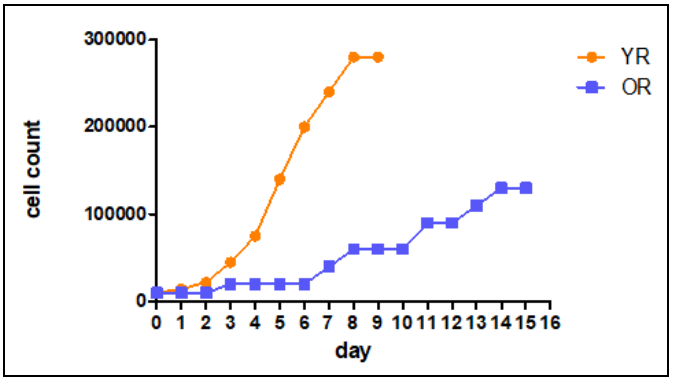


FIG 2A: PLOT OF GROWTH CURVES FOR YOUNG AND OLD BMMSC. Shows the growth curve of young and old rat bone marrow mesenchymal stem cells.

The young rat MSCs showed a lag phase of 24 hours after which the cells multiplied exponentially for 7 days and reached a plateau thereafter. Confluency was reached on day 9, and the average number of cells at the confluent stage of culture were 2.82×10^5 cells per well of 24 well plate P< 0.01) (P value summary: **)

The growth characteristics of old rat MSCs differed significantly from the young rat. They, too, showed a lag phase of about 1 day. The rate of cell growth was however much slower and required almost 16 days ($p<0.001$) to reach to confluency. The cell count at the confluent stage was 1.3×10^5 cells. The population doubling time calculated using the formula described earlier was 1.36 +1 days for YRMSC and 3.54 + 1 days for ORMSC respectively.

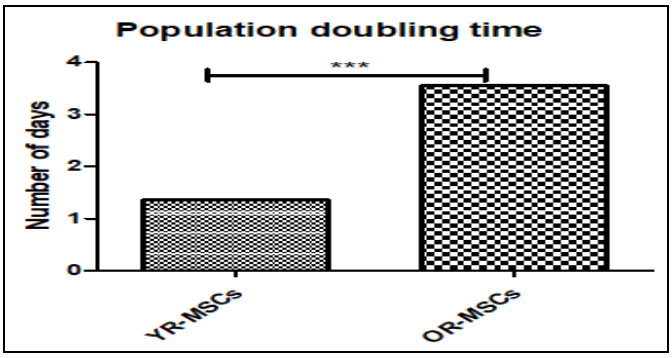


FIG. 2B: GRAPH OF POPULATION DOUBLING TIME OF YOUNG RAT AND OLD RAT MESENCHYMAL STEM CELLS. Shows the graph of population doubling time in days of young rat and old rat mesenchymal stem cells.

The population doubling time estimated from the graph was 1.36±1 days for young rat MSCs, whereas the old rat MSCs tended to double their population in the average of 3.54 ±1 days. ($p<0.05$) **Fig. 2B**. Population doubling time value was thus significantly lower than that for old rat MSCs indicating the more rapid rate of proliferation of YR-MSCs than OR-MSCs.

Adipogenic Differentiation: The young and old rat MSCs were cultured in adipogenic differentiation medium for 7 days and subsequently stained with oil-red-O dye. The oil red O from cells was eluted with isopropanol, and absorbance measured at 500 nm wavelength. **Fig. 3A** shows the photographs of oil red O stained undifferentiated control cells and differentiated adipocyte cells of young and old rat-derived MSCs. The percent adipocyte differentiation of young and old rats is given in **Fig. 3B**.

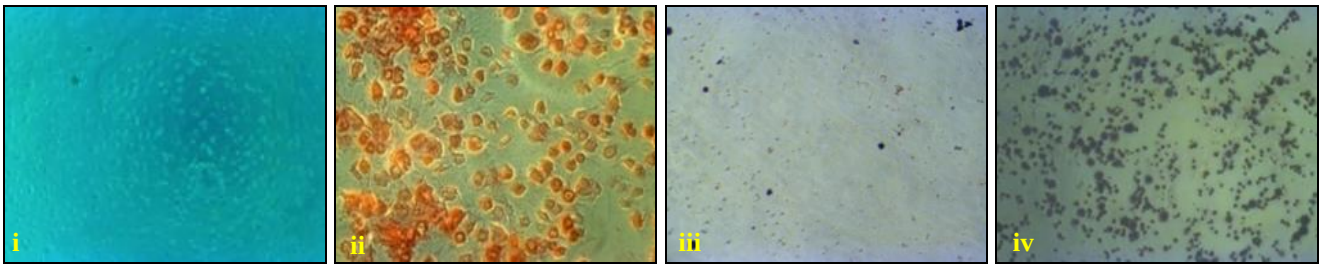


FIG. 3A: ADIPOCYTE DIFFERENTIATION OF YOUNG RAT BM-MSCs AND OLD RAT BM-MSCs. A- I & ii shows the images of cell control and adipocyte differentiated MSC of young rat respectively. Fig 3 (A) iii & iv shows images of cell control and adipocyte differentiated MSC of old rat, respectively

Both YR-BM-MSCs and OR-BM-MSCs were differentiated into adipogenic lineages. About 54% of young rat MSCs differentiated to adipocytes. Differentiation was expected to decline in the case of old MSCs. However, a significant proportion, *i.e.*, almost 97% of cells differentiated to adipocytes indicating much enhancement in adipogenic differentiation (P > 0.01) by old cells.

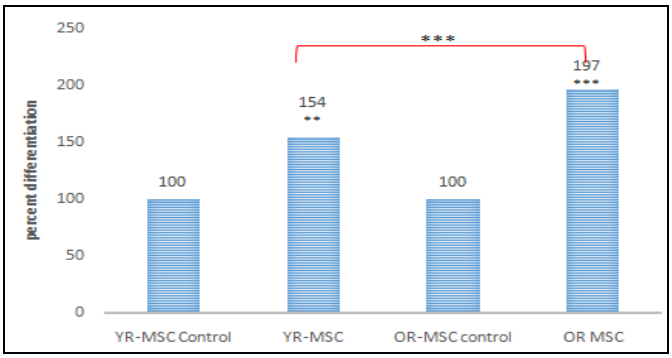


FIG. 3B: GRAPH OF ADIPOCYTE DIFFERENTIATION OF YOUNG RAT AND OLD RAT MESENCHYMAL STEM CELLS. Shows the fold increase/decrease in adipogenic differentiation in YR-BM-MSCs and OR-BM-MSCs

Chondrocyte Differentiation: Differentiation to chondrocytes was mediated by treatment with a chondrocyte differentiation medium for 14 days. The cells were then stained with alcian blue stain and photographed. The stain was eluted using guanidine hydrochloride, and absorbance was read at 600 nm wavelength.

The images of cell control and the chondrocyte differentiated young and old MSC are given in **Fig. 4A (i–iv)**. The graphical representation is presented in **Fig. 4B**.

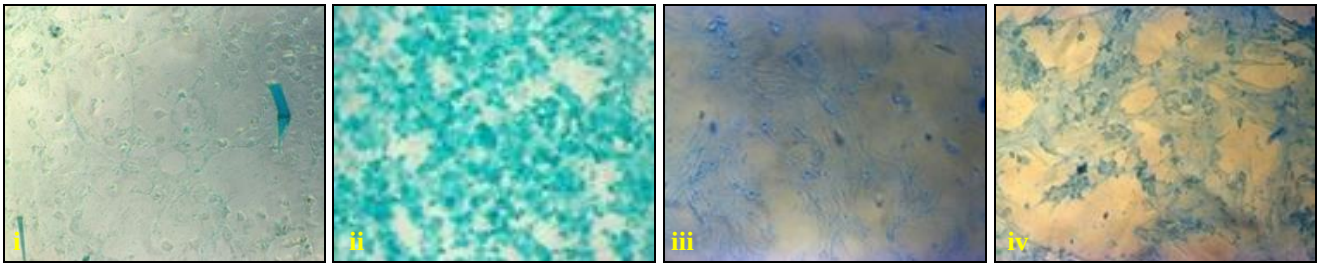


FIG. 4A: CHONDROCYTE DIFFERENTIATION OF BONE MARROW MESENCHYMAL STEM CELLS ISOLATED FROM YOUNG AND OLD RATS. Fig 4 (A) I & ii shows the images of cell control and chondrocyte differentiated MSC of young rat respectively. iii & iv shows images of cell control and chondrocyte differentiated MSC of old rat, respectively.

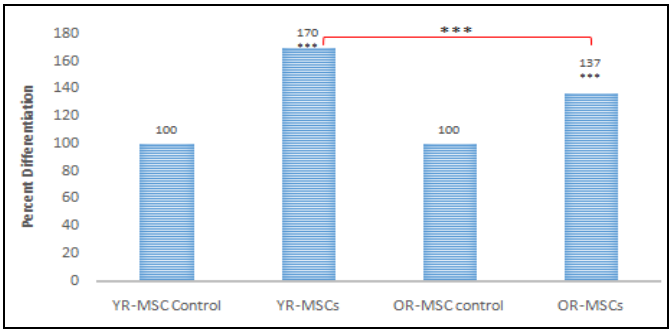


FIG. 4B: GRAPH OF PERCENT CHONDROCYTE DIFFERENTIATION OF YOUNG RAT AND OLD RAT BM MSCs. Fig. 4(B) shows the increase/decrease fold in chondrocyte differentiation of young rat and old rat BM MSCs

Young rat MSCs readily differentiated into chondrocytes. About 70 % of young cells were found to differentiate into chondrocytes. In contrast, only 37 % of old cells could achieve that. Significant reduction ($P < 0.001$) of aged MSCs potential to differentiate into chondrocytes was thus noted.

Osteocyte Differentiation: Incubation of MSCs in osteocyte specific medium for 21 days and subsequent staining with alizarin red gives an estimate of osteocyte differentiation. Absorbance at 405 nm of stain eluted with acetic acid and ammonium hydroxide could measure the dye taken up by differentiated cells, which in turn quantifies the extent of osteocyte differentiation of MSCs. **Fig. 5A** shows the images of cell control and osteocyte differentiated young and old cells. The optical density readings determined 43 % differentiation of young cells versus 29% of old cells **Fig. 5B**. Significant reduction in osteocyte differentiation potential of aged MSC as compared to the young were thus observed ($P < 0.01$).

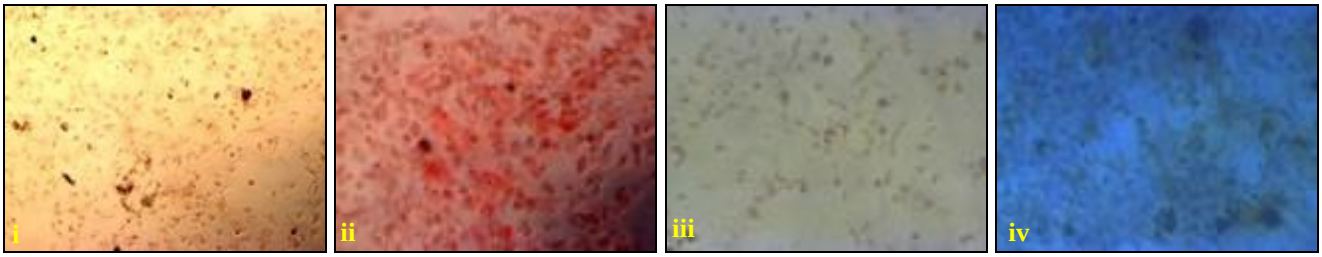


FIG. 5A: OSTEOCYTE DIFFERENTIATION OF YOUNG RAT AND OLD RAT BONE MARROW MESENCHYMAL STEM CELLS. Fig. 5 Ai & ii shows the images of cell control and osteocyte differentiated MSC of young rats, respectively. iii & iv shows images of cell control and chondrocyte differentiated MSC of old rat, respectively

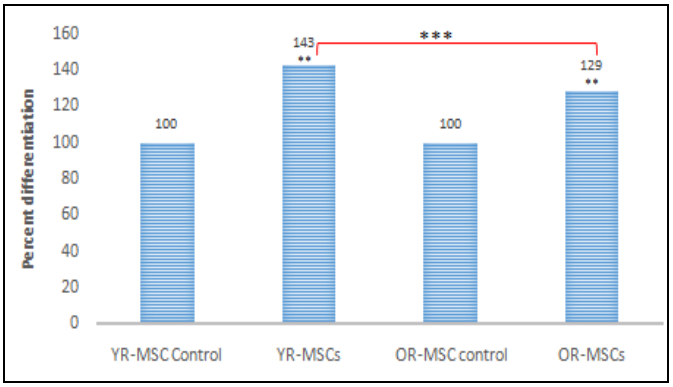


FIG. 5B: GRAPH OF OSTEOCYTE DIFFERENTIATION OF YOUNG RAT AND OLD RAT MESENCHYMAL STEM CELLS. Fig. 5(B) shows the increase/decrease fold in osteocyte differentiation of young rat and old rat BM MSCs

SA-β-Gal Staining: Increase in β – galactosidase enzyme activity is associated with senescence hence serves as a marker for the determination of cellular senescence.

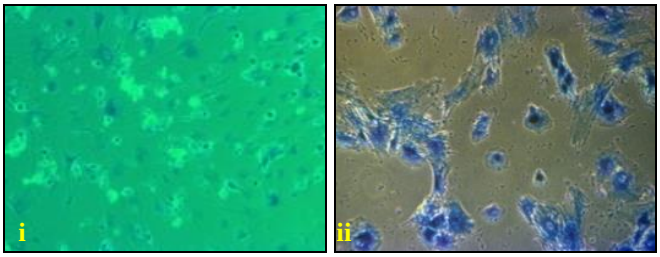


FIG. 6A: SENESCENCE ASSOCIATE BETA GALACTOSIDASE STAINING OF YOUNG RAT AND OLD RAT BM MSCs. Fig. 6(A) i-ii shows the image of SA-β-gal stained young and old rat MSCs respectively

Cells stained in blue colour indicate SA-β-gal positive cells. They were quantified *via* microscopic examination and images were captured.

As many as 64 % of cells were SA -β-galactosidase positive and thus senescent in aged MSCs as compared to only 14% in young MSCs. A significant increase (P<0.001) in senescent cells in older MSCs were thus observed. The morphology of old rat MSCs was prominent in microscopic examinations. They were more enlarged and flattened than those of young rat MSCs.

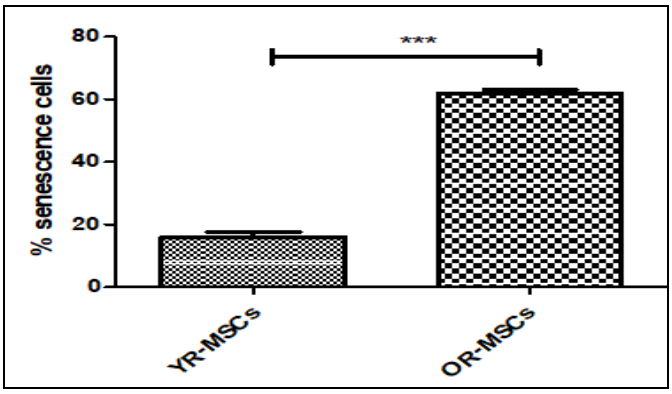
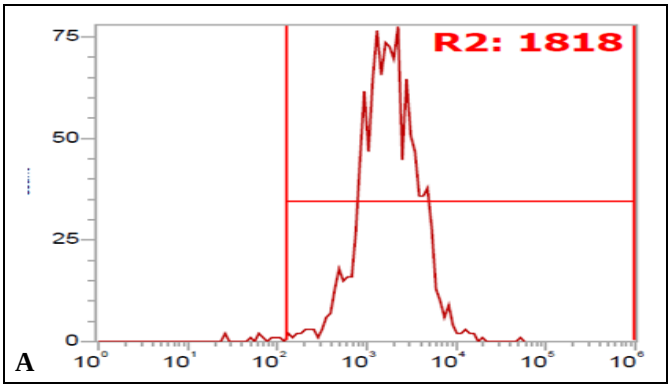
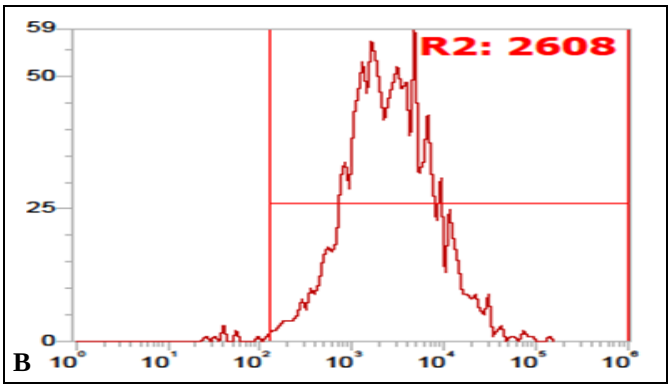


FIG. 6B: QUANTIFICATION OF SENESCENCE OF YOUNG RAT AND OLD RAT BM-MSCs. Fig. 6(B) shows the Quantification of senescence of young rat MSCs and old rat MSCs, as detected by SA-β-gal staining

ROS Staining: Accumulation of reactive oxygen species (ROS), mostly free radicals such as nitric oxide, superoxide anions and hydroxyl radicals in cells, is a major contributing factor for loss of homeostasis, which in turn is a cause of cellular senescence. Determination of ROS is an important parameter for determining senescence. The ROS values were quantified by fluorescence analysis of dichlorofluorescindiacetate (H_2DCFDA) stained cells. The cells were treated with H_2DCFDA for 25 minutes and assessed for fluorescence at 488 nm excitation and 525 nm emission in a flow cytometer.

Quantification of intracellular ROS levels was performed by fluorescence-activated cell sorting analysis of stained cells to obtain mean fluorescence intensity. The histogram and quantification of the ROS generation in young and old MSCs are described in **Fig. 7A, B,** and **C**.





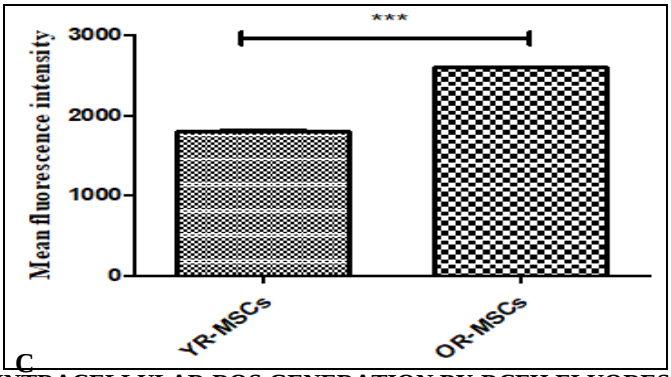


FIG. 7: QUANTIFICATION OF INTRACELLULAR ROS GENERATION BY DCFH FLUORESCENCE. Fig 7 (a) and (b) show the DCFH fluorescence intensity in young and old rat BM-MSCs, respectively. Fig 7(C) shows the comparison of YR-MSCs and OR-MSCs ROS levels

The mean fluorescence intensity (MFI) of young rat MSCs was 1818, and old rat MSCs was 2608. Much higher DCFH fluorescence intensity indicating intracellular ROS levels were thus observed in old rat MSCs compared to young rat MSCs ($P < 0.001$), indicating more generation of intracellular ROS. It is known that oxidative stress is a prime cause of aging. It leads to DNA damage, protein damage and mitochondrial dysfunction, which triggers the process of senescence.

RT-PCR Analysis:

TABLE 1: DELTA VALUES of $p16^{INK4a}$, p21 AND TELOMERASE GENES

Sample	$P^{16INK4a}$	P^{21}	telomerase
YR-MSCs	28.084	22.623	27.196
OR-MSCs	32.330	21.406	22.444

The table above shows the respected delta values of expressed $p16^{INK4a}$, p21 and telomerase genes in young rat and old rat mesenchymal stem cells.

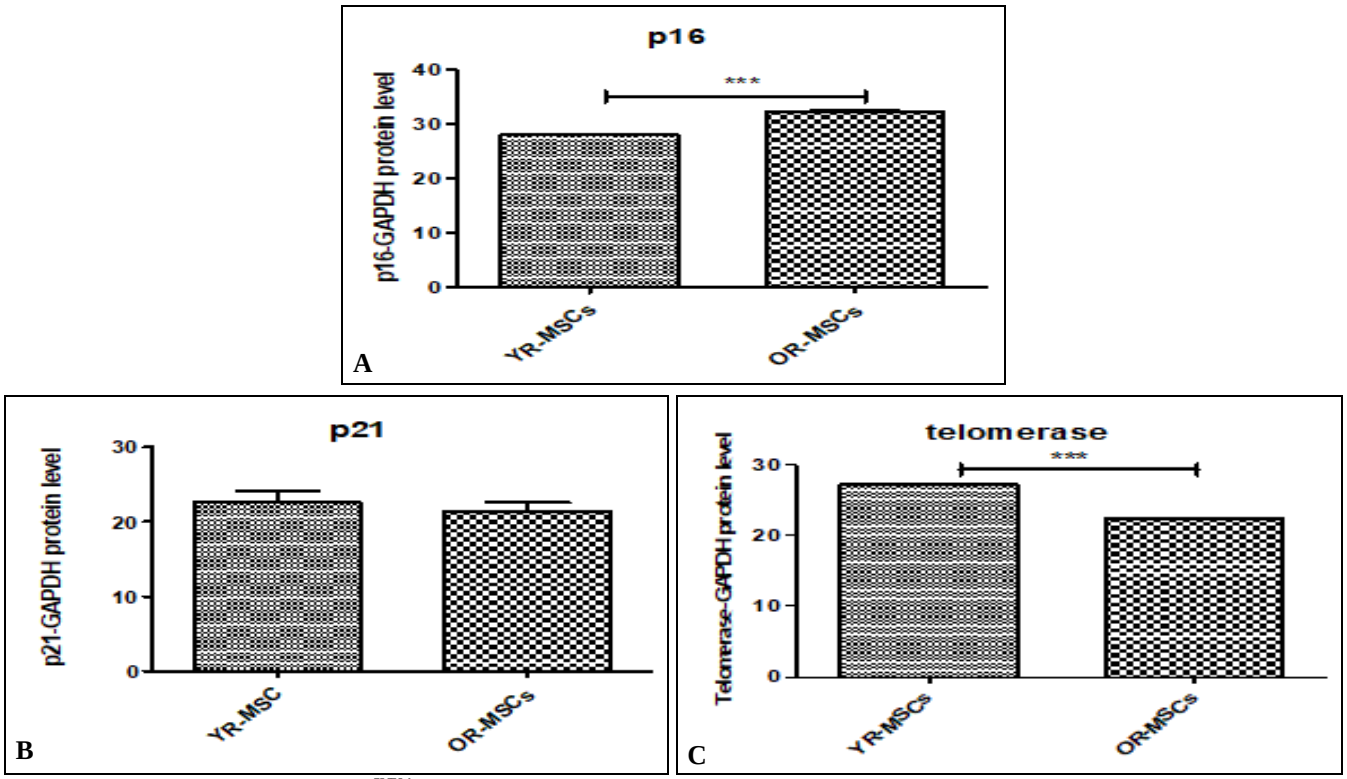


FIG. 8: RT-PCR ANALYSIS OF $p16^{INK4a}$, p21 AND TELOMERASE GENES IN YOUNG RAT AND OLD RAT MESENCHYMAL STEM CELL. Fig 8 (a) shows the expression level of $p16^{INK4a}$ in young rat and old rat mesenchymal stem cells. Fig 8 (b) shows the p21 gene expression level in young rat and old rat mesenchymal stem cells. Fig 8(c) shows the telomerase gene expression level in young and old rat mesenchymal stem cells.

Senescence in MSC are associated with alteration in gene expression. Progressive telomere attrition leading to non-repairable damage to DNA is a confirmed cause of replicative senescence. The enzyme telomerase prevents telomere erosion and induces telomere elongation by continuous

restoration of the lost TTAGGG repeats at the chromatin termini. Down-regulation of telomerase is suggestive of telomere attrition and subsequent senescence. Another genes correlating with aging process are $p16^{INK4a}$ and p21. The code for proteins involved in control of G1 to S transition. $p16^{INK4a}$ expression gradually increases with aging in most mammalian tissues. Earlier studies have shown that elevated $p16^{INK4a}$ expression is suggestive of growth retardation and increased senescence [9].

The expression of telomerase, p21 and $p16^{INK4a}$ genes in young and old MSC cultures were determined using real-time PCR. The expression levels are shown in **Fig. 8A, B,** and **C**. The down-regulation of telomerase and significant up-regulation of $p16^{INK4a}$ and p21 were noted in old MSCs.

DISCUSSION: *In-vitro* culture of mesenchymal stem cells is one of the foremost and important step in regenerative medicine and tissue engineering applications. Allogenic MSCs obtained from perinatal tissues having maximum functional capacity may not be possible in many applications due to unsuitability or unavailability reasons. One of the barriers encountered in the expansion of autologous MSCs is a decline in their functions with increasing age. MSC also shows this feature *in-vivo* in later age and is considered as a major cause of the decline in maintenance of tissue homeostasis, organ failure, and degenerative diseases [10]. In the present study, we have compared the parameters of MSC isolation, frequency, rate of cell proliferation, differentiation potential, and senescence of cells isolated from young and old rats.

The present study demonstrated that age has a significant negative impact on MSC functions. The decline in function could be seen right from the primary culture isolation stage. The frequency of cells was drastically reduced in the primary culture of old MSCs. Approximately 50% recovery of aged MSCs was observed in primary culture as compared to young MSCs. The cells exhibited almost 7 days of lag phase and also determined varied morphology. They appeared more enlarged and flattened, having less association with neighboring cells. Their rate of proliferation was much slower and required almost 27 days to form a monolayer. The young MSCs, on the other hand, adhered by the third day and overcome the lag phase on the fourth day. They showed typical fibroblast-like morphology and multiplied exponentially in log phase. The cells achieved confluency on15^{th} day. The cell count at the confluent stage was much higher than old cells. The yield of cells in primary culture, their adherence to the substrate and ability to proliferate was thus drastically reduced in aged rats. The seeding density for subsequent passages was 1×10^4 cells, which is higher than the cells isolated in primary culture. An increase in seeding density in subsequent passages improved the lag period and rate of cell growth significantly. The PDT for the two groups of cells in subsequent passages were 1.36 ± 1 and 3.54 ± 1 days, respectively. The young rat MSCs had much higher growth potential and were passaged upto 17 passages with a similar yield of cells. The old rat cells in at least four attempts that we tried could not be cultured beyond 5 passages. The population doubling time, cell yield, and self-renewal ability of cells isolated from old rats were thus drastically reduced. A number of earlier studies undertaken on MSCs isolated from various sources have reported similar findings. Qiao Yi *et al.*, (2017) [11] found that Dental pulp stem cells (DPSC) from young donors exhibited more powerful proliferation ability than old donors. There are few reports, however, showing no correlation of PDT with an age of MSC [12].

The multilineage differentiation capacity of the two groups was assessed to evaluate the impact of age on cells. The percentage of cells capable of undergoing osteogenic differentiation declined from about 43% in young to about 29% in aged cells (32.55% decrease). The chondrogenic potential also determined a decrease from 70% in young to about 37 % in old rats (48.7% decrease). On the contrary, the adipogenic potential showed a substantial increase in old cells. The adipogenic differentiation potential increased from 54% in young cells to as high as 97% in old cells (79% increase). The results clearly revealed the reduced efficiency of old MSCs to differentiate into osteocytes and chondrocytes. In contrast, as many as 97% of cells could be differentiated to adipocytes. Most studies on bone marrow-derived MSCs have shown a reduction in the multilineage potential of MSCs [13, 14].

Our study showed a decline in osteogenic and chondrogenic potential with enhancement in age. They, however, displayed a high tendency to differentiate into adipocytes [15].

An important marker of cellular senescence is an increase in SA-β-Gal activity. It reveals an increased activity of the lysosomal β-D galactosidase enzyme which is induced at suboptimal pH 6.0 created due to increased lysosomal activities in these cells [16]. The percentage of SA-β-Gal positive cells in aged MSCs was 64% in the present study compared to only 14% in young cells. If the data is normalized, then the old cells represent about 50% SA-β-Gal positive cells. It may be correlated directly to the occurrence of senescent cells in culture. It thus indicates the presence of a substantial amount of senescent cells in aged MSCs.

Accumulation of reactive oxygen species is a major causative factor for senescence. Increased frequency of generation of free radicals such as nitric oxide (NO), superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^·$) due to abnormalities in mitochondrial metabolism are noted in senescent cells. Increased activity of cytochrome C oxidase, NADH dehydrogenase, and decreased mitochondrial membrane potential observed in senescent cells also suggest mitochondrial dysfunction [17]. Reduced activity of enzymes such as superoxide dismutase that catalyzes the dismutation of superoxide into oxygen and H_2O_2detected in senescent MSCs also contributes to an increase in ROS [18]. The ongoing oxidative stress causes DNA damage, protein damage, and mitochondrial dysfunction in these cells. They lead to aberrant folding, aggregation, and accumulation of proteins in MSC, contributing to senescence. A prominent increase in ROS level in the old cells was observed in our study. In terms of percentage, it amounts to a 50% increase in old cells as compared to young MSC. It correlates with the earlier findings of this study showing about 50% decline in proliferative ability and presence of 50% of SA-β- Gal positive and thus senescent cells.

It is reported that the changes in stem cells are associated with progressive attrition of telomeric DNA sequences caused due to inactive telomerase enzymes. Downregulation of the telomerase gene is an indicator for telomere shortening. Our data show a decrease in expression of the telomerase gene and an increase in cell cycle checkpoint kinase p16 ^{INK4A} and p21 gene expressions. Previous studies have shown that p16 ^{INK4A} gene expression increases with age in various tissues.

The p16 ^{INK4A} locus on chromosome 9 encodes two tumor suppressor proteins, p16 ^{INK4A,} and p14, which are involved in growth arrest, cellular senescence, and apoptosis in most mammalian tissues. It controls the G1 to S transition by binding to $CDK_{4/6,}$ inhibiting its kinase activity and thereby preventing Rb phosphorylation [19]. P21 is a nuclear protein that indicates senescence-associated cell cycle arrest [20]. The p16 ^{INK4A} positive cells show growth retardation and increased activity of SA-β-gal. The knockdown of p16 ^{INK4A} expression reversed senescent features of MSCs, suggesting that p16 ^{INK4A} plays an essential role in the aging process [21]. The increased expression of p16 ^{INK4A} p21 genes and decreased telomerase gene in the present study clearly indicate the presence of replicative senescence in MSCs derived from old rats.

To summarize, the present study has determined growth characteristics of MSC isolated from old rats. It could define the lag/log periods, passage intervals, and cell yield at confluency in comparison to young cells. About 50 % decrease in frequency, PDT, and cell yield was observed. A similar proportion of SA-β-gal positive cells suggesting the presence of 50 % senescent cells in MSC isolated from old rats.

The decline in osteogenesis and chondrogenesis, increase in adipogenesis, accumulation of ROS, and altered expression of senescence specific genes telomerase p16 ^{INK4A} and p21were the important features of old rat cells. The study has provided baseline information for defining a methodology that will retrieve MSCs retaining optimal functions. The key target to pursue include determining strategies that could prolong the growth and differentiation capabilities of MSCs and/or design media formulation that promotes MSC functions for their suitable application.

CONCLUSION: Mesenchymal stem cells isolated from 4 – 6 weeks young and 53 -56 weeks old rats were compared for cell growth, differentiation, and senescence parameters. Reduced frequency of cells

in primary culture, population doubling time, and cell yield at confluency were shown by old cells. Their differentiation to osteocytes and chondrocytes was much less as compared to young cells. They, however had a high potential to differentiate into adipocytes. Significant increase in SA-β-gal activity, ROS levels, and senescence specific proteins p16 ^{INK4A} and p21 genes were important features of old cells. The study helped in determining the *in-vitro* tissue culture characteristics of young and old MSCs. They may be of value in defining the strategies for enhancing the growth and differentiation capacities of autologous cells.

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