Resveratrol Antiaging Impact on Bone Marrow-Derived Mesenchymal Stem Cells in A Duration-Dependent Manner

Original Article

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ABSTRACT

Introduction: Aging is a natural life event associated with progressive cellular and biological dysfunction. It is a cumulative process that ends with impairment of the general function of the biological system. Stem cells are unique cells with behavioral diversity making them intriguing candidates for regenerative therapy.
Resveratrol (RSV), a nonflavonoid polyphenol phytoalexin with a stilbene structure, was first extracted from the root of white hellebore, and it was widely used in traditional Chinese medicine.
Aim of Work: We tried to postulate if resveratrol had therapeutic effects on aged MSCs and whether these effects were duration dependent or not
Materials and Methods: We achieved our study on bone marrow-derived mesenchymal stem cells (BM-MSCs) derived from 15-month aged rats. We used 40 rats; subdivided into four equal subgroups; (A) control, (B) received resveratrol for one week, (C) received resveratrol for two weeks, and (D) received resveratrol for one month. Morphological changes were tracked, surface marker expression was measured, and various parameters of proliferation kinetics were used; colony-forming unit (CFU), population doubling time (PDT) "3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)", "DNA index" and budding uninhibited by benzimidazole-related 1. (BubR1) immunocytochemical staining were implemented.
Results: We reached significant results in improving morphological changes, proliferation kinetic parameters, immunophenotypic flow cytometric CD90 marker expression, DNA index, and anti BubR1antibody immunocytochemical staining even after two weeks of treatment.
Conclusions: Resveratrol had flourishing effects of BM-MSCs on aging cells. It promoted growth, proliferation, improved viability, established genetic stability, and improved MSC aging parameters documented in marked duration-dependent patterns.

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Key Words: Ageing, bubR1, MSCs, resveratrol.

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INTRODUCTION

Aging is an intricate fact known to be affected by various aspects like diet, lifestyle, environment, heredity, and disease. It slows down the biological machine that keeps tissues alive, helps with immunity, and maintains people healthy[1].

Bone marrow has classically been described as a unique tissue composed of two main systems, giving rise to two different cell lineages; the hematopoietic tissue proper and the associated supporting stroma. Evidence of assumed stem cell plasticity giving rise to the wide spectrum cell lineages has been established[2].

Marrow stromal cells are the stem/progenitor cells of skeletal tissues, in addition to their original involvement in the creation of the hematopoietic milieu. Furthermore, studies show that marrow stromal cells have a wide range of potential neural tissue[3]. Several fields of marrow stromal cell biology gained appeal and interest, having diverse implementary therapeutic potential, particularly for tissue repair and regeneration[4]. Stem cells carry an intrinsic challenge being "lifelong cells." The hazard of developing genomic damage is increased with aging[5]. The acquisition of DNA damage in adult stem cells can be influenced by various factors, including telomere attrition, free radicals, defective autophagic machinery, and improper proteostasis[6].

Aging impacts two axes of mesenchymal stem cells (MSC): a reduction in the bone marrow MSC pool and a preference for development to adipocyte rather than osteoblast, resulting in osteoporosis[7]. Also, MSC aging is detrimental to some non-skeletal tissues, in particular the hematopoietic system. All these factors result in MSC fitness compromise and decline sufficiency to organ regeneration[8,9].

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Resveratrol is a natural plant extract that dominates wide both protective and therapeutic capabilities for acute or chronic injury in multiple tissues due to its antioxidative, anti-inflammatory, and anti-cancer properties[10].

Resveratrol (RSV) proved to preserve mitochondria in its optimized dose, act as an antioxidant, and establish cellular metabolic homeostasis, ending in acting as an antiaging agent and getting recommendations to retard aging-related disorders[11]. Resveratrol is an anti-inflammatory and antioxidant supplement that activates the sirtuin 1 (SIRT1)[12]. Sirtuin 1 (SIRT1) is a histone deacetylase protein of class III that plays a role in cellular processes, including aging and energy metabolism[13,14].

Our literature highly shines resveratrol as an antiaging agent and its possible role in not only the retarding physiological deterioration, but can also reverse BM-MSCs aging parameters and upregulate MSC age-related profiles.

AIM OF THE WORK

This experimental study aimed to establish whether RSV ingestion positively affect in-*vitro* cultured senescent bone mesenchymal stem cells (BMSCs) derived from 15 months aged rats. We aimed to correlate between RSV and morphological changes of cultured cells, proliferation rate, proliferative kinetics parameters, immunophenotypic CD marker expression, DNA index representing aneuploidy cells, and anti BubR1 antibody immunocytochemical staining. We tried to postulate if resveratrol had therapeutic effects on aged MSCs and whether these effects were duration dependent or not.

MATERIALS AND METHODS

Chemicals

- Trans-resveratrol (30 g powder), (Sigma company), USA.
- Culturing supplies including Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), trypsin, penicillin-streptomycin, and phosphate-buffered saline (PBS) included: NaCl 8.0 g, KCl 0.2 g, KH2PO4 0.24 g, and Na2HPO4 1.44 g in 1 L Milli-Q water (pH 7.4, sterilized and stored at four °C) were purchased from Gibco company, Egypt. Plastic supplies, including Flasks, Petri dishes, coverslips, falcons, Pasteur pipettes, variable-sized pipettes, 6well plates, were obtained from Lonza, Egypt.
- Methanol (Mallindkrodt, Hazelwood, USA)
- Crystal violet (Sigma Aldrich, Egypt).
- Antibodies for stem cell characterization: CD34, CD31, and CD90 flow cytometry,

CD90 Monoclonal Antibody (OX-7), FITC Catalog # MA1-81491,Thermo Fischer, UK).

CD34 Monoclonal Antibody (1H6), FITC Catalog # MA1-81639, Thermo fisher, UK.

CD31 Monoclonal Antibody (PECAM-1), PE Catalog # 12-0311-83, eBioscience (San Joes,USA).

For a DNA index: TM plus DNA kit (BD; San joes, CA95131 USA).

- BubR1 primary Ab for immunocytochemical detection of Bubr1 checkpoint protein (Goat anti-Rabbit IgG (H+L). Secondary Antibody, from (Chongqing biopsies company, China).

Animal groups

A total number of 40 female albino Sprague-Dawley rats, 15 months aged group weighing between 200-300g. They were obtained from the animal house of Assiut University. Animals were maintained under controlled laboratory conditions of humidity, temperature, and light: dark cycle. Rats were fed normal rat's chow and allowed free access to water. All animal procedures were approved by the Institutional Ethics Committee of Assiut University. Animal suffering was minimized. Resveratrol was given orally (through an intragastric tube) daily at a dose of 2.5 mg/kg diluted in 0.5ml phosphate buffer saline[15].

The animals were divided into the following subgroups:

A: The control group consisted of ten rats who were given phosphate buffer saline (0.5 ml).

B: 10 rats received resveratrol for one week.

C: 10 rats received resveratrol for two weeks.

D: 10 rats received resveratrol for four weeks

At the end of the experiment, we sacrificed animals and dissected in a sterile dissecting room[16].

Isolation of bone marrow-derived mesenchymal stromal cells

Rat bone marrow samples were obtained. We sacrificed animals by cervical dislocation, dissection was done after alcohol splashing, then we removal of soft tissue to get out fore and hind limb long bones under safety cabinet, then flushing the bones was dine using DMEM. After that, centrifugation of DMEM with bone marrow for 10 minutes at 1500 Rpm was done. Then washing was done twice using sterile 5% (PBS penicillin-streptomycin). Lastly the cell pellet was cultured using a complete medium (DMEM with 10% FBS and 1% penicillin/ streptomycin, and incubated in 5% CO2 at 37 °C[17].

Cell cultivation (Cell plating)

Removing floating cells after 48 hours, and the medium was changed twice a week until the monolayer of adherent cells reached 80-90% confluence. The mesenchymal population was identified based on its plastic adherence capacity, morphological criteria, and flow cytometric marker analysis[17].

Flow cytometric characterization of in *vitro* cultured BM-MSCs

The following monoclonal antibodies, CD90, CD31, and CD34, were used. Conjugated with Fluorescein isothiocyanate (FITC) or Phycoerythrin (PE), they have used for flow cytometric characterization of MSCs. In brief, cells were trypsinized after reaching 90% confluence and re-suspended in PBS. 1 x 104 cell suspension was incubated in the dark with labeled antibodies at room temperature for one hour. Negative controls of FITC corresponding immunoglobulins were used. Then samples were washed and mounted immediately after staining. A total of 10,000 events were acquired, at low speed, after the cytometer was compensated and calibrated. FACS Canto II flow cytometer was used for sample acquisition, and FACS Diva 6.1 software was used for the analysis[18].

Cell proliferation analysis

Using a solution of trypsin and EDTA (0.25 percent), subculture was enacted, and proliferation kinetic parameters were implemented. Cell viability and the number of recovered cells were evaluated using a hemocytometer and quantified using the Trypan Blue exclusion test[19].

Colony- forming unit fibroblast (CFU-F) assay

It measures the capability of MSCs of different experimental groups to perform colonies. CFU was investigated by culturing 1000 cells in triplicate of a 6-well plate for 14 days in a 5% CO2 humidified incubator at 37 °C. Cells were washed twice using DPBS -Mg2+, - Ca2+ and fixed for 20 minutes using 100% methanol at room temperature, followed by staining with 3% crystal violet. Using tap water, cells were washed until the dishes became colorless. The dishes were air-dried for several minutes after inversion downwards on a clean paper. CFU was estimated as following; CFU = (total number of stained colonies >2 mm / initial number of cells) x 100%[20].

Colony surface area (CSA)

We estimated the previous colony surface area and statistically analyzed them.

Population doubling time (PDT)

In the log-phase/exponential phase, we determined the average time it takes for a cell population to double in number. From each group, cells were plated as 10^4 cells/cm2 in 25 cm2 culture flask (N0), so N0 refers to the number of cells at the initial plating time. When reaching 90-95% confluency, trypsinization and counting cells were done using a hemocytometer (N). N is the number of cells at the end of the cultivation phase. Population doubling time calculated according to the equation; PDT=culture time (CT)/PDN. (PDN, population duplication number, was calculated according to the formula: PDN=log NxN0×3.31.We calculated them in a triplicate manner[21].

Cell proliferation assay (MTT)

To detect cell viability, a colorimetric MTT assay was achieved. The MTT assay replaced the radioactive tritiated thymidine incorporation assay. It is the first widely accepted method to measure cell proliferation. After four days of culture in triplicate of 6well plate, MTT solution was added, and the cells were incubated in a 5% CO2 humidified incubator at 37 °C for four hours. Then the formazan solubilization solution was added, and the absorbance was detected at 650 nm[22].

Cell Cycle and DNA analysis

About 10.000 cells in suspension were centrifuged at 400x g at room temperature (20-25°C) for 5 minutes. After discarding the supernatant, the sediment cell pellets were suspended and incubated for 10 minutes in 250 µl of trypsin buffer (Solution A) at room temperature. After that, 200 µl of trypsin inhibitor was added with RNAse buffer (Solution B) without aspiration of solution A and incubate at room temperature for 10 minutes. Then, 200 µl of cold propidium iodide (PI) was added and gently mixed into each tube. Finally, the tubes were incubated in the dark at (2-8 °C). Samples are now ready to be analyzed by flowcytometry. The identification and quantification of DNA aneuploidy/euploidy states and the cell cycle phase calculations were accomplished with the aid of the ModFit LT software[23].

Flow cytometry was performed to calculate the cell percentages in each cell cycle phase and measure the DNA index (DI) and the aneuploid /euploid DNA content ratio. The cytometric estimation of the degree of DNA content abnormality is commonly represented by DI, which is the ratio of the Go/G1 peak channel of the aneuploidy cells with normal DNA diploid cells. A normal diploid DNA content is represented by a DNA Index of 1.0, whereas deviation in cellular DNA content values other than 1.0 indicates DNA aneuploidy or polyploidy. When at least two separate G0/G1 peaks are detected, the occurrence of DNA aneuploidy was reported[24].

Immunocytochemical study

Starting cell seeding on sterilized glass coverslips in 6 well plates. The media were removed. Paraformaldehyde (PFA) (4 percent 0.3 ml per well) was used to fix the cells for 15 minutes at 4°C. The cells were washed twice in PBS for one minute each time. Then, for 30 minutes at room temperature, Triton x 0.4 percent was applied. PBS washes three times for three minutes each time. The goat serum solution was administered at room temperature for 1.5 hours. Anti-Bubr1 staining was done at a dilution of 1:100 at 4◦c overnight. Then washing with PBS 3 times each was for 5 minutes. At room temperature, the biotinylated secondary antibody was administered for 1–5 hours. PBS was used for washing three times for five minutes each time. DAB solution was applied for 5-10 minutes. Washing was done with PBS 3 times each for 2 minutes. Lastly, mounting was done[25].

Statistical analysis

Results were obtained from at least three independent

experiments. Data were expressed as mean ± standard deviation. Using GraphPad Prism 5 statistical software (GraphPad, La Jolla, CA) and Excel software (Microsoft, Redwood, WA), Differences were analyzed by Student's t-test after one-way analysis of variance[21] (ANOVA). Differences were considered significant when the probability values (*P*) were less than 0.01.

RESULTS

Morphology of BM-MSCs during in vitro culture

BM-MSCs were observed by an inverted microscope. The attached cells were checked 24-48 hours after isolation and kept up to 80-90%confluence. Adherent cells were observed and tracked 2days, 4days, one week, and ten days post culture.

Two days post culture: Morphological changes and proliferation rate were reported. Relatively larger rounded cell aggregation appeared in group B in comparison to group A. Some spindle-shaped fibroblast-like cells appeared in group C and progressive increase in group D (Figures1 a,b,c,d).

Four days post culture Morphological changes and proliferation rate were reported. A progressive increase in fibroblast-like spindle-shaped cells was observed in groups B, C&D in comparison to group A (Figures 2 a,b,c,d). Heterogeneous cell population appeared in group C; some were spindle-shaped cells, others were branched and characteristic syncytial-like arrangement of attached cells (Figure 2c). Whereas in group D, there were numerous attached fibroblast-like spindle-shaped cells and few branched cells (Figure 2d).

One week post culture: Morphological changes and proliferation rate were reported. Group A showed few mononuclear cells, aggregated rounded cells, spindle-shaped and branched cells (Figure 3a). Proliferating spindle-shaped cells appeared in groups B&C, displaying syncytial-like arrangement, and flattened sheath-like cellular arrangement was observed (Figures 3 b,c). In group D, cells exhibited highly expanded confluent cells in an anastomosing arrangement. Granular finely stippled cytoplasm could be observed in some cells (Figure 3d).

After ten days of culture, We observed similar changes to one-week post culture but in an advanced manner and more confluent cellular network (Figure 4 a,b,c,d).

A highly magnified previous image; regarding group A, some cells exhibited hypertrophic changes. Central condensed nuclei with clumped chromatin and intracytoplasmic vesicles could be demonstrated. Clear halo around some cells could be seen. Some cells showed budded vesicles (Figure 5a). In group B, fibroblast-like and branched cell morphology with small hypertrophic flat cells could be identified. Some nuclei were centrally located vesicular nuclei; others were condensed with clumped chromatin. Relatively fewer intracytoplasmic microvesicles and finely stippled cytoplasm with clear halos around cells were easily demonstrated (Figure 5b). In group C, well-demarcated syncytium, centrally located vesicular nuclei, and finely stippled cytoplasm were clearly demonstrated. Clear halos around some cells could be seen (Figure 5c).

Regarding group D, highly expanded cells with euchromatic vesicular nuclei and granular finely stippled filamentous cytoplasm could be demonstrated. Cells were like a complete well-defined sheet. Some cells were bi-nucleated (Figure 5d).

Immunophenotypic characterization

Flow cytometric analysis of CD90, CD31and CD34 marker expression of BM-MSCS of subgroups A, B, C and D.

Cells stained positively for CD90 and negatively for CD31 (Figures 6 a,b,c,d).

(Table 1) Statistical analysis of flow cytometric results of CD90, CD31, and CD34 marker expression showed a significant increase in positively expressed CD90 in both groups C&D compared to group A as well as group B, while a significant increase in negatively expressed CD31 in group D compared to groups A, B &C.

Proliferation kinetics of BM-MSCs

We calculated the colony surface area (CSA), colony-forming unit (CFU), Population doubling time (PDT), and cell viability (MTT) to confirm previous subjected results

a. Colony surface area (CSA): showed a significant increase in colony surface area in group C compared to groups A&B. Also, a significant increase in colony surface area in group D compared to groups A, B&C (Figure 7).

b. Colony-forming unit percentage (CFU): showed a significant increase in colony-forming unit percent in group C compared to groups A&B. Significant increase in group D compared to both groups A&B (Figure 8).

c. Population doubling time (PDT) showed a significant decrease in PDT in groups B, C&D compared to group A. Significant decrease in groups C&D compared to group B (Figure 9).

d. Cell viability (MTT): showed a significant increase in MTT in groups B, C &D compared to group A. Significant increase in MTT in groups C&D compared to group B (Figure 10).

Cell Cycle and DNA analysis

Cell cycle and DNA analysis for different experimental groups of A, B, C&D. Representative charts of flow cytometric analysis using propidium iodide (PI) for different groups. Group A showed that most cells had normal diploid DNA with some aneuploidy cells (Figure 11a). Group B showed most cells had normal diploid DNA with some DNA aneuploid ones. However,

aneuploidy cell proportion is less than the control group (Figure 11b). Group C showed most cells had normal diploid DNA with relatively less percent of DNA aneuploidy (Figure 11c). Group D showed normal diploid DNA with no DNA aneuploidy (Figure 11d).

This result was confirmed by (Table 2), which represented a progressive decrease of the percentage of aneuploid cells down to zero after four weeks of resveratrol treatment. This referred to DI (proportion) to be progressively increased to be 1 after four weeks resveratrol treatment.

Immunocytochemical staining of anti BubR1 antibody

BM-MSCs were stained by anti BubR1 antibody and observed by light microscope in groups A, B, C and, D.

Group A showed a mild positive nuclear reaction and a moderate positive cytoplasmic immunocytochemical reaction for BubR1 in some cells. Others exhibited negative nuclear reactions (Figure 12a). Group B showed a positive cytoplasmic immunocytochemical reaction for BubR1. Some cells exhibited positive nuclear reactions; others exhibited negative nuclear reactions (Figure 12b). Group C showed strong positive cytoplasm immunocytochemical reaction for BubR1 cytoplasm, whereas nuclear reaction was negative (Figure 12c). Group D showed strong positive cytoplasm immunocytochemical reaction for Bubr1 cytoplasm, whereas nuclear reaction was negative (Figure 12d).

Morphometric analysis of positively reacted cells for anti BubR1 antibody expression of groups A, B, C&D were reported and statistically analyzed in (Table 3).

It revealed a significant increase in the number of positively reacted cells in Group C compared to A. Moreover, group D showed a significant increase in positively reacted cells compared to groups A, B&C.

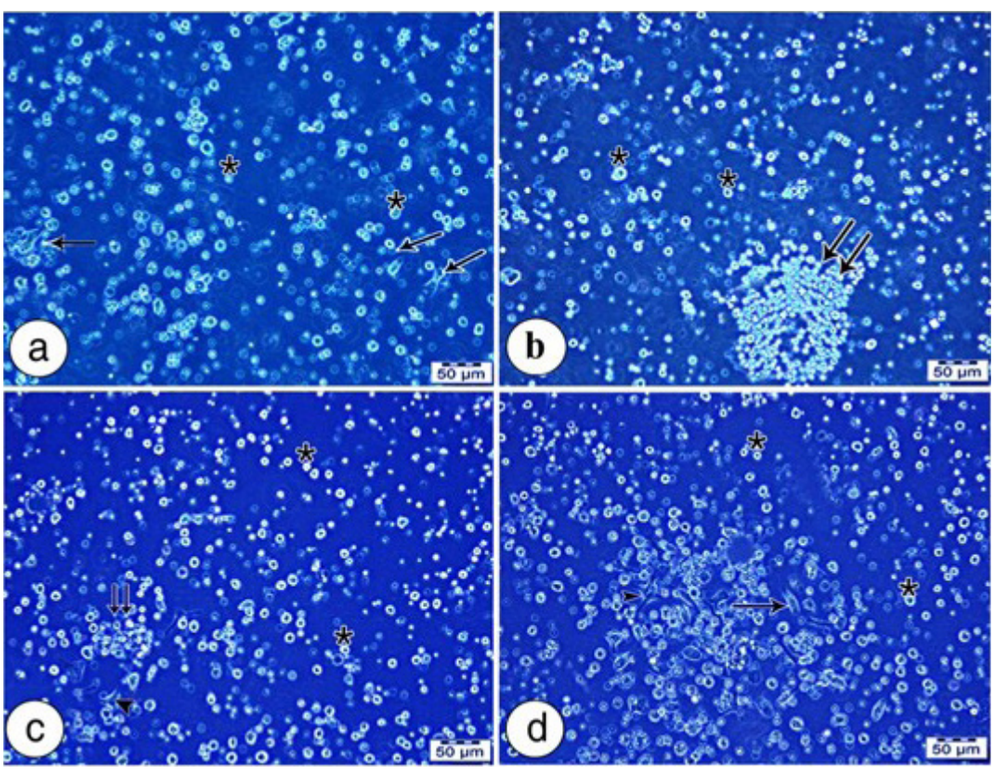

Fig. 1: Photomicrographs of BM-MSCS 2 days post culture. A (a) shows scattered mononuclear cells (star), cell aggregation (double arrow) and initial cell elongation and inconspicuous spindle shaped cell formation (arrow). B (b) shows mononuclear cells (star) as well as circumscribed attached round cell (double arrow) with no observed spindle shaped cells. C (c) shows scattered mononuclear cells (star), more attached branched cells (arrow head) as well as round cell aggregation (double arrow). D (d) shows few scattered mononuclear cells (star) more attached fibroblast like spindle shaped cells (arrow) and few branched cells (arrow head). (Inverted microscope X400)

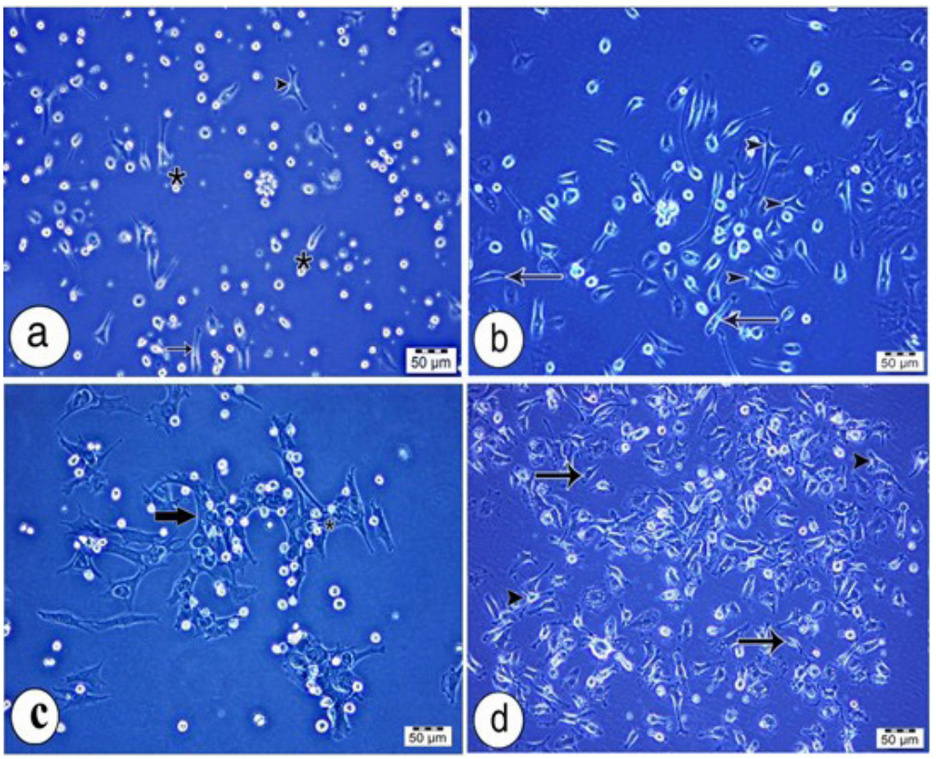

Fig. 2: Photomicrographs of BM-MSCS 4 days post culture; A (a) shows some mononuclear cells (star), some branched cells (arrow head) as well as few number of spindle shaped cells (arrow). B (b) shows numerous fibroblast like spindle shaped cells (arrow) and some branched cells (arrow head). C (c) shows syncytial like arrangement (thick arrow), and finely stippled cytoplasm (star). D (d) shows the most defined and numerous attached both fibroblast like spindle (arrow) and branched cells (arrow head). (Inverted microscope X400)

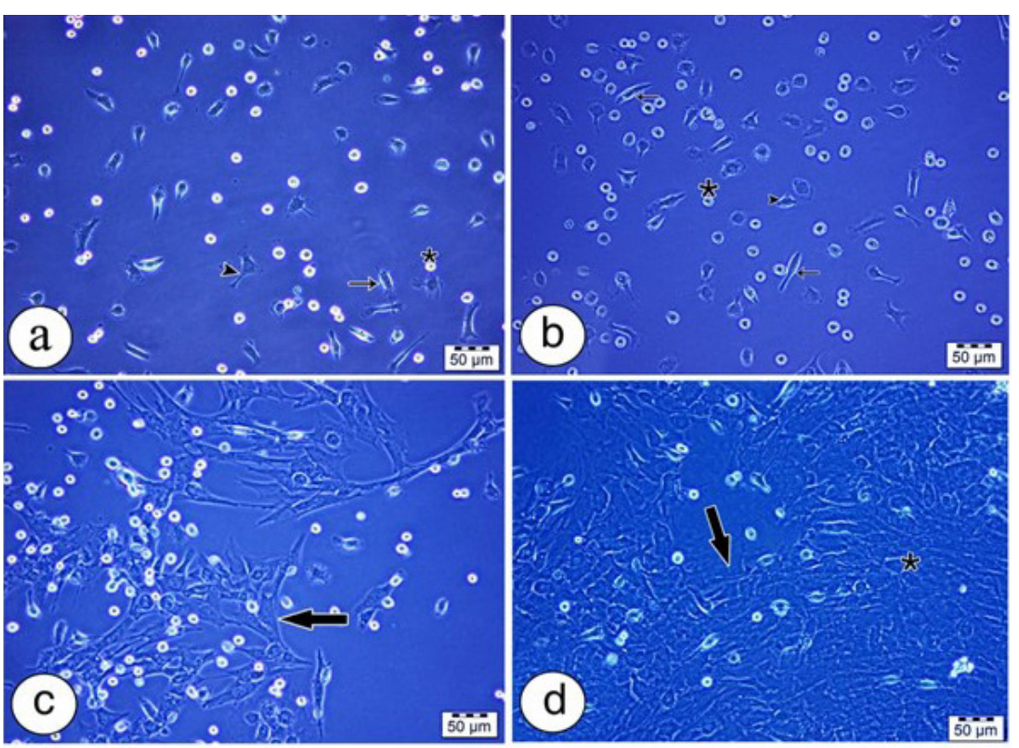

Fig. 3: Photomicrographs of BM-MSCS one week post culture; A (a) shows scattered mononuclear cells (star) , spindle shaped (arrow) and some branched cells (arrow head). B (b) shows some mononuclear cells (star), numerous spindle shaped (arrow) and a few scattered branched cells (arrow head). C (c) shows multiple spindle shaped cells displaying syncytial like arrangement (thick arrow), flattened sheath like cellular arrangement. D (d) shows highly expanded cells, in anastomosing arrangement (thick arrow). Finely stippled cytoplasm can be observed in some cells (star). (Inverted microscope X400)

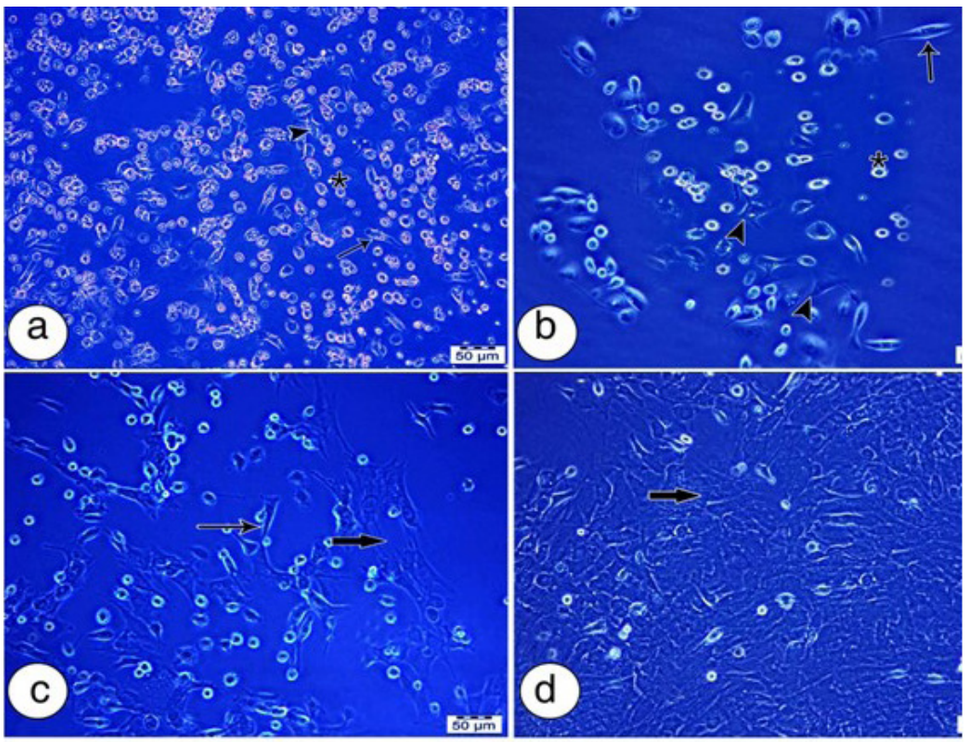

Fig. 4: Photomicrographs of BM-MSCS 10 days post culture; A (a) shows some mononuclear cells (star), multiple spindle shaped cells (arrow) and few branched cells (arrow head). B (b) shows fibroblast like spindle shaped cells (arrow) with some branched cells (arrow head). Some scattered mononuclear cells (star). C (c) shows syncytial like organization (thick arrow) of spindle shaped cells (arrow). D (d) shows a full confluent network of spindle shaped cells (thick arrow). (Inverted microscopeX400)

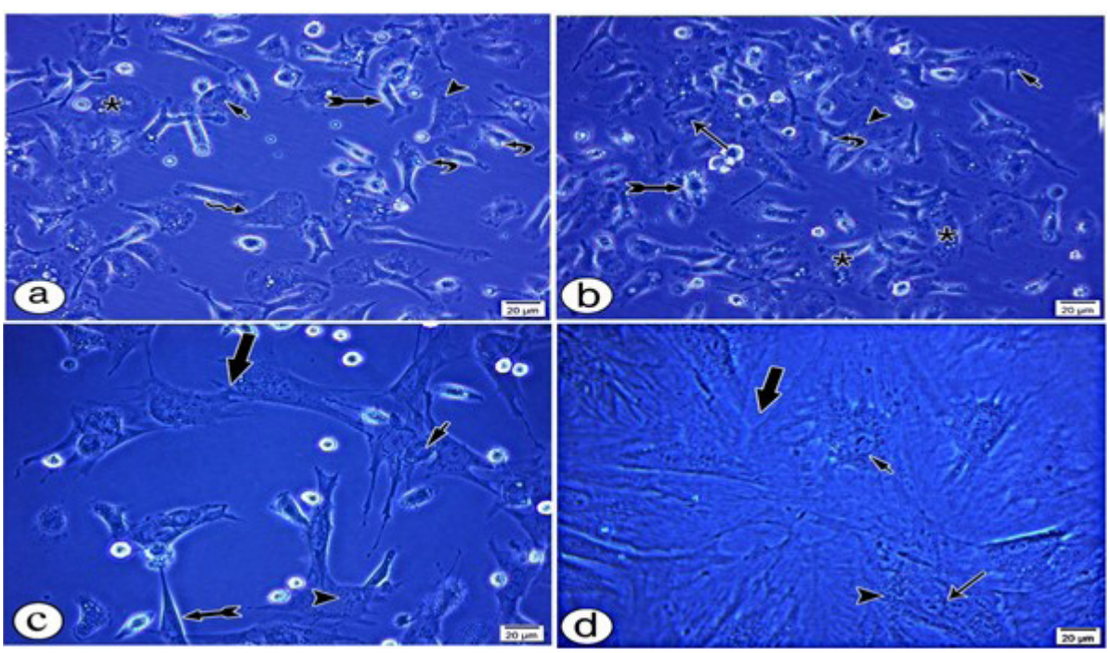

Fig. 5: A higher magnified previous image; A (a) shows both spindle shaped and branched cells. Some cells are hypertrophied (wavy arrow). The nucleus is condensed with clumped chromatin (short arrow). Intra cytoplasmic micro vesicles (star) and finely stippled cytoplasm (arrow head) can be demonstrated. Clear halos around cells can be seen (tailed arrow). Some cells exhibit budded vesicles (curved arrow). B (b) shows both fibroblast like and branched cell morphology. Some nuclei are centrally located and vesicular (short arrow), others are condensed (arrow). Intra cytoplasmic microvesicles (star) and finely stippled cytoplasm (arrow head) are easily demonstrated. Clear halos around cells can be seen (tailed arrow). Some cells exhibit budded vesicles (curved arrow). C (c) shows well demarcated syncytium (thick arrow), centrally located vesicular nuclei with minimal chromatin clumps and finely stippled cytoplasm (arrow head) are shown. Clear halos surrounds some cells can be seen (tailed arrow). D (d) shows highly expanded cells, finely stippled cytoplasm (arrow head), some nuclei are centrally located and vesicular (short arrow). Some cells exhibit double nuclei (arrow). Cells are like complete well defined sheet (thick arrow). (Inverted microscope X1000)

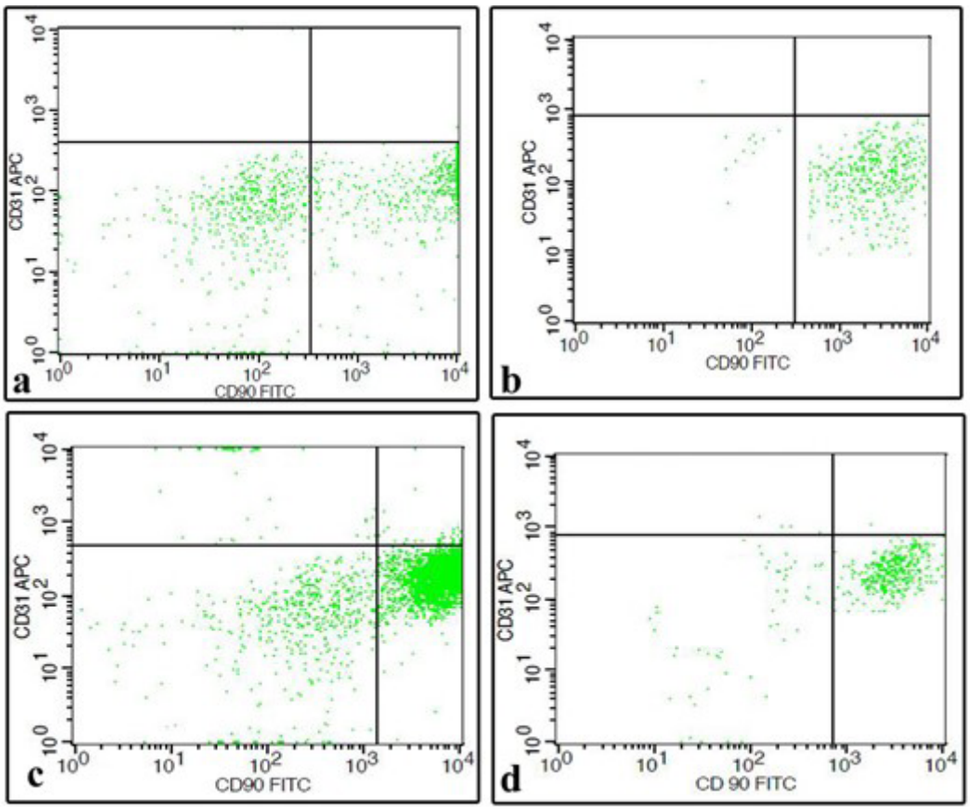

Fig. 6: Expression of MSCs surface markers measured by FACS analysis in BM-MSCs. The cells showed positive expression of CD90 and negative expression of CD31 (FACS analysis).

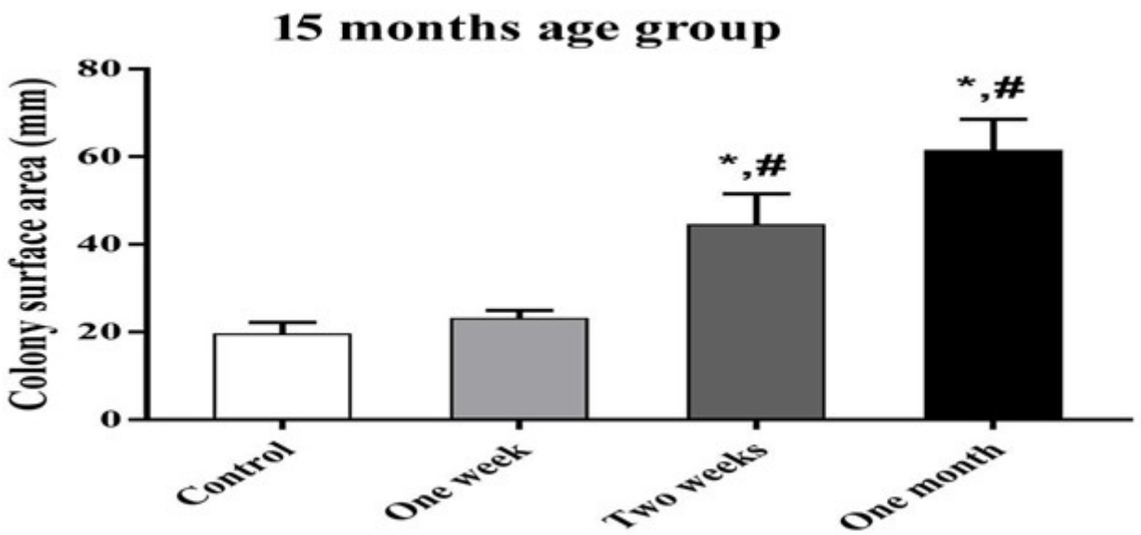

Fig. 7: Statistical analysis of colony surface area exhibited by proliferating cultured cells captured simultaneously amongst all groups (4 days post culture). Significant increase in colony surface area in both two week and one month(C&D) resveratrol treated group compared to both control and one week resveratrol group (A&B).
Data are expressed as mean ±SD.
*Significantly different from control group $P<0.05$.
#Significantly different from one week group $P<0.05$.

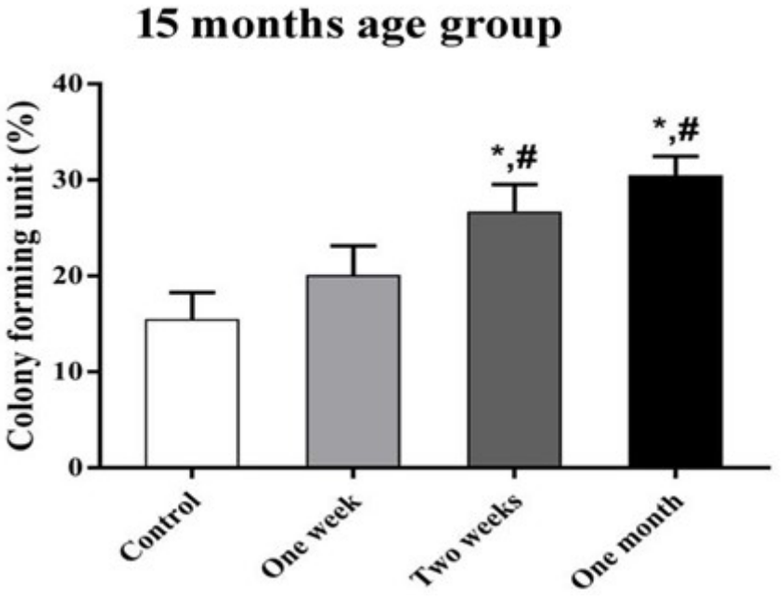

Fig. 8: Colony forming unit assay of more than one year aged-group of in *vitro* cultured BM-MSCs ; Colonies >2 mm were detected when cells plated in triplate of six-well plate; Bars represent mean ± SD. Significant difference between passages is analyzed by one-way ANOVA test. Significant increase in colony forming unit percent in both two week and four weeks resveratrol treated group(C&D) compared to both control and one week resveratrol group (A&B). BM-MSCs: bone marrow-derived mesenchymal stromal cells.
*Significantly different from control group $P<0.05$.
#Significantly different from one week group $P<0.05$.

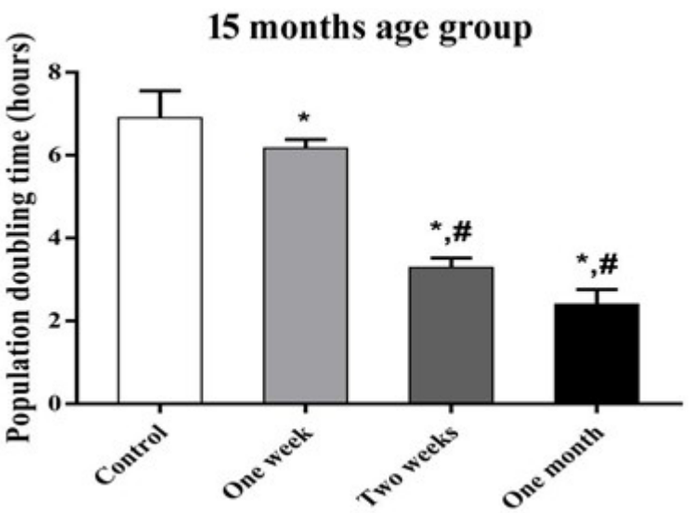

Fig. 9: Population doubling time of different experimental groups of BM-MSCs; Bars represent mean ± SD. Significant difference between passages is analyzed by one-way ANOVA test. Significant decrease in PDT in all resveratrol treated groups (B,C&D) compared to control group (A). This significance also is evident in both two week and four weeks resveratrol treated groups(C&D) compared to one week resveratrol treated group (B). BM-MSCs: bone marrow-derived mesenchymal stromal cells
*Significantly different from control group $P<0.05$
#Significantly different from one week group $P<0.05$.

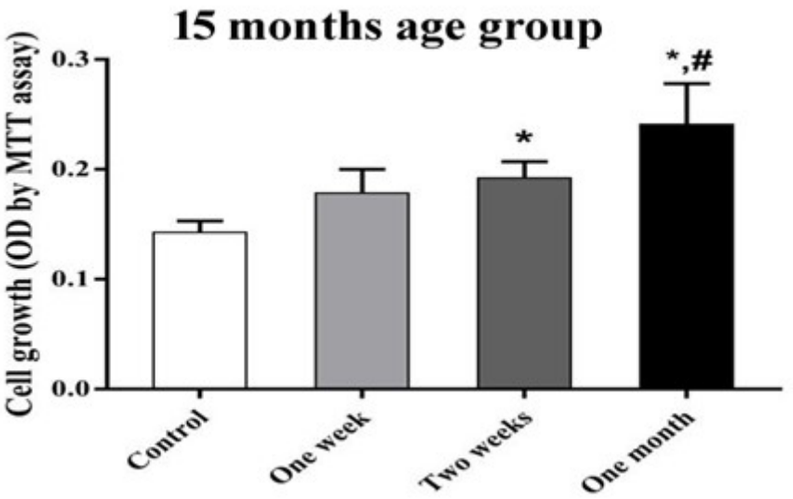

Fig. 10: Cell viability assay of more than one year aged-group of in *vitro* cultured BM-MSCs; viability of BM-MSCs was evaluated using MTT assay and the representative absorbance of the cells measured at 650 nm is shown. Bars represent mean ± SD. Significant difference between groups is analyzed by one-way ANOVA test, whereas, significant increase in MTT in both two weeks and four weeks resveratrol treated groups (C&D) compared to control(A). This significance is also evident in four weeks resveratrol treated group (D) compared to one week resveratrol treated group (B). BM-MSCs, bone marrow-derived mesenchymal stromal cells; MTT, 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide
*Significantly different from control group $P<0.05$.
#Significantly different from one week group $P<0.05$

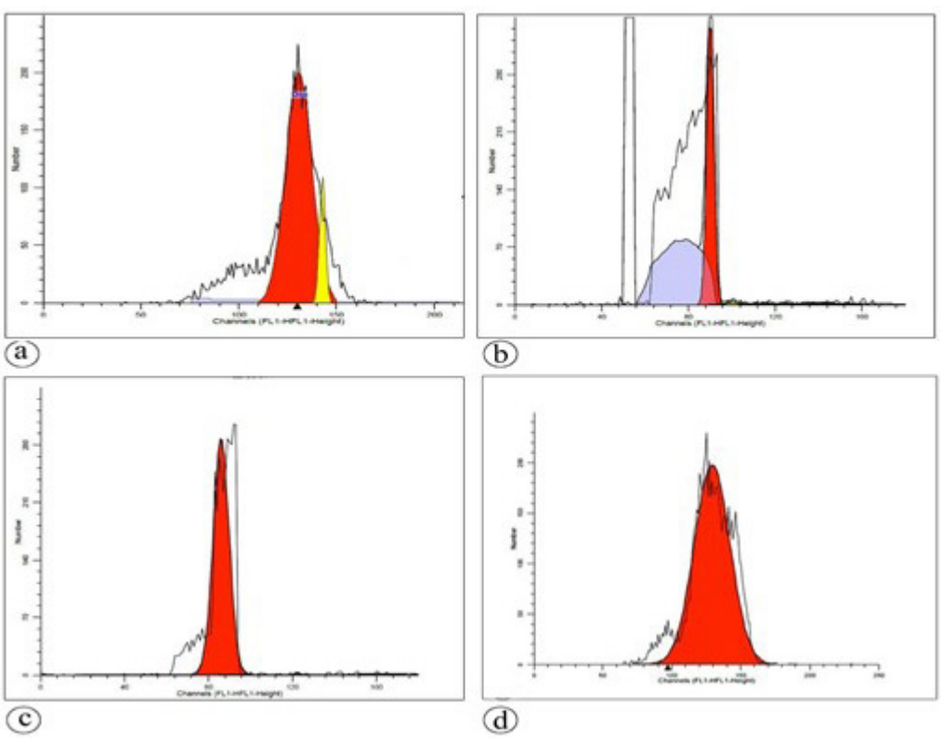

Fig. 11: Cell cycle and DNA analysis for the different passages of in *vitro* cultured BM-MSCs. Representative charts of flow cytometric analysis using propidium iodide (PI) for different groups. Group A (a) shows most cells to have normal diploid DNA with very small percent with DNA aneuploidy. Group B (b) shows diploid DNA with fewer aneuploidy cell percent. Group C (c) shows that aneuploidy cells are fewer than above described groups. Group D (d) shows full diploid cellular percent.

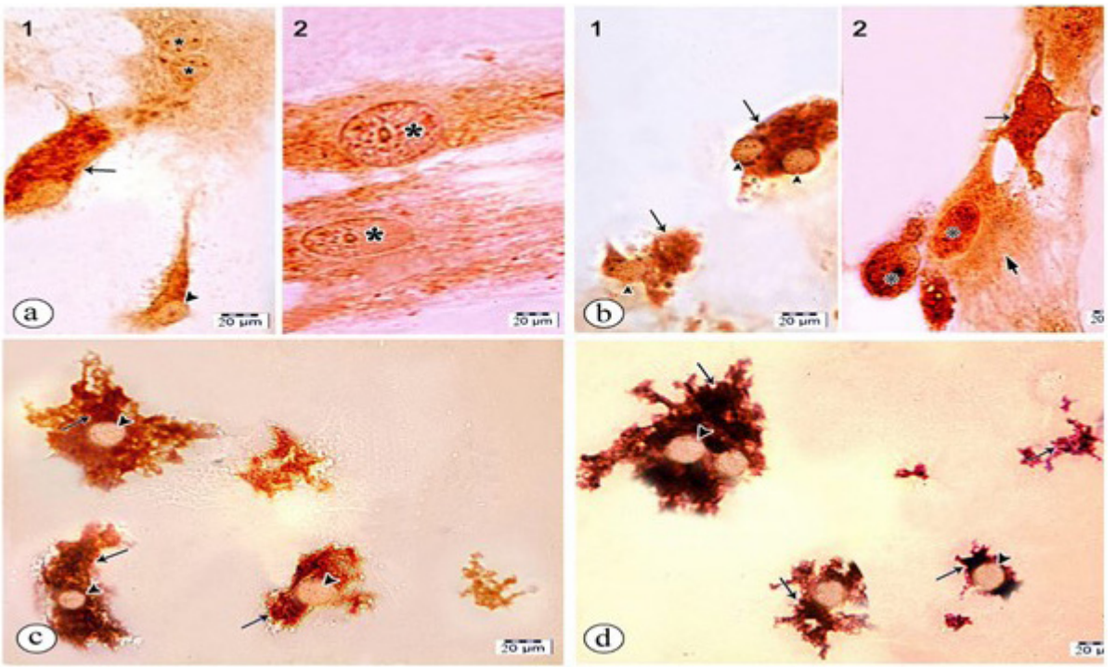

Fig. 12: Photomicrographs of BM-MSCS of 15 month-aged group demonstrating immunocytochemical expression of anti BubR1 Ab; Group A (a) (1&2) show mild positive nuclear reaction (star), moderate positive cytoplasmic reaction (arrow). Many negatively stained nuclei can be detected (arrow head). Group (B) (1&2) shows mainly moderate positive cytoplasmic reaction (arrow). Some nuclei are negatively stained (arrow head) ; others are moderate positively stained (star). Group C (c) shows intensively reacting cells with deep brown cytoplasm ; reaction is homogenous rather than granular (arrow), negatively stained nuclei are shown (arrow head). Group D (d) shows intensively reacting cells with deep brown cytoplasm; reaction is homogenous rather than granular (arrow). Negatively reacting nuclei can be seen (arrow head) (Anti BubR1 Ab reaction X1000)

Table 1: Statistical analysis of CD90 and CD31 markers expression in BM-MSCS show significant increase in positively expressed CD90 in both two weeks and four weeks resveratrol treated groups (C&D) compared to control group (A) as well as one week resveratrol treated group (B), while significant increase in negatively expressed CD31 in four weeks resveratrol treated group (D) compared to the whole control , one week and two week resveratrol treated groups (A,B&C).

	Control	One Week	Two Week	One Month
CD90	49.85±4.71	65±0.77	81.03±4.41*#	85.62±4.10*#
CD31	0.40±0.073	2.01±0.43	1.51±0.44	22.01±3.98*#$

Data are Expressed as mean ± SD
* Significantly different from control group $p<0.05$
# Significantly different from One Week group $p<0.05$
$ Significantly different from Two Week group $p<0.05$

Table 2: Cell cycle distribution. Control group revealed 87.11 % of cells in normal diploid and only 12.89% aneuploidy , progressive reduction in the aneuploidy percent; 96.83% diploid and only 3.17% aneuploidy in one week resveratrol treated group; 98.85% diploid and small percent 1.15% aneuploidy in two weeks resveratrol treated group and100% diploid cells in four weeks resveratrol treated group. So DI of control group was 0.87. It was 0.96 for one week resveratrol treated group, 0.98 for two weeks resveratrol treated group and 1 for four weeks resveratrol treated group

In*vitro* culture MSCS	DNA ploidy mode	Frequency of cells at G0/ G1 phase (mean value%)	Frequency of cells at S phase (mean value %)	Frequency of cells at G2/M phase (mean value%)
Control group	DNA diploid 87.11% DNA aneuploidy 12.89%	DNA diploid 61.52% DNA aneuploidy 89.68%	DNA diploid 0.00% DNA aneuploidy 0.00%	DNA diploid 38.48% DNA aneuploidy 10.61%
One week resveratrol group	DNA diploid 96.83% aneuploidy 3.17%	DNA diploid 97% DNA aneuploidy 0.00%	DNA diploid 0.37% DNA aneuploidy 0.00%	DNA diploid 1.93% DNA aneuploidy 0.00%
Two week resveratrol group	DNA diploid 98.85% aneuploidy 1.15%	DNA diploid 91.23% aneuploidy 100%	DNA diploid 0.00% DNA aneuploidy 0.00%	DNA diploid 8.77% DNA aneuploidy 0.00%
One month resveratrol group	DNA diploid 100% aneuploidy 0.00%	73.31% (or 49.9%) DNA aneuploidy 0.00%	(2.54%) DNA aneuploidy 0.00%	(47.55%) DNA aneuploidy 0.00%

Table 3: Statistical analysis of anti BubR1 antibody expression of 15 months aged-group of the control, one week, two weeks and four weeks resveratrol treated groups of in *vitro* cultured BM-MSCs showing a significant increase in number of positively reacted cells in both two-weeks and four weeks resveratrol treated groups(C&D) compared to control group (A) and in four weeks resveratrol treated group (D) compared to one week and two weeks resveratrol treated groups (B&C).

	Control	One Week	Two Week	One Month
Number of positive cells	10±1.58	15±1.72	20±3.16*	30±4.74*#

DISCUSSION

Aging is a complex cumulative biological process characterized by MSC functional decline, tissue homeostasis impairment, and a delay in the replacement and turnover of injured cells. This results in compromised renewability profiles in tissues. The effective application of MSCs in regenerative medicine and stem cell-based therapy necessitates a basic understanding of MSC aging[26].

Resveratrol (RSV) is a "natural phytoalexin" with rejuvenating effects in experimental animal models, tissues and organs, and, most importantly, stem cells[27,28]. Tseng *et al*. (2011) and Shakibaei *et al*. (2012) declared that Resveratrol implicated MSCS considerably. It influenced cell viability, osteogenic differentiation, and paracrine secretion in *vitro*[29,30].

This research aims to study the chronological aging impact on MSC isolated from aged rats and how the oral resveratrol administration can significantly improve and / reverse aging profiles. We found that BM-MSCs derived from aged rats exhibited a relatively slow growth rate and proliferation, with different morphological features compared to those isolated from resveratrol-treated groups.

We observed that BM-MSCS derived from the aged control group attained the same confluence as resveratrol treated group but took a longer culture period. That reflected the relatively little proliferation potential of MSCs in aged animals; these findings might be explained by the impaired microenvironment of these cells with the progress of age[31].

The slow proliferation rate might be explained by general aging theory/theories of cells as imbalances between antioxidants and free radical existence. Thus oxidative stress developed with a hazard of nucleic acid oxidation, DNA damage, and reduced adhesion of MSCs. Furthermore, telomere length reduction with aging might be implicated in the impaired proliferation potential observed in the present study. These suggestions were raised after recent data correlated age with telomere length[32].

After chronic exposure of MSCs to high doses of growth-stimulating substances, downregulation of growth factor receptors was expected. Therefore, resistance to these factors eventually resulted in cellular senescence. In contrast, MSCs in *vivo* were slowly cycling cells. As a result, telomere erosion seemed unlikely to be the primary cause of their in *vivo* aging[33].

Similarly, the transplantation of MSC from aged rats had a lower success rate. In clinical trials, donor age affected therapeutic potency[34]. Additionally, aging might have a deleterious impact on MSCs' multi-lineage differentiation capabilities[5,35,36].

The stem cell niche was considered as an in *vivo* regulatory microenvironment where stem cells reside. MSC niches in BM were primarily in the perivascular and bone lining locations. It was found that perivascular BM regions were more oxygenated than endosteal regions[1,37].

The observed hypertrophied and flat cells with granulated vesicular and filamentous cytoplasm in the control group could be explained by having a slow proliferation rate with more accumulated cell inclusions and /or end product metabolism in comparison to small spindle-shaped cells. These findings could be explained by the difference in the microenvironment in the aging process that might have a detrimental effect on the stem cell niche in aged animals.

Some researchers noticed that compared to earlier passages, the relative area of BM-MSCs increases over 10-fold in later passages, which is connected with an increase in actin stress filaments[38,39].

Within MSC cultures in adult animals, Prockop and his colleagues discovered two morphologically distinct subpopulations: small, rapidly self-renewing multipotent cells and more mature slower replicating bigger cells[40].

Moreover, Colter *et al*. (2001) accepted time-based morphological transition of cultured MSCs from thin (small), spindle-shaped cells (considered stem cells or early progenitors) to wider (larger) spindle-shaped cells (looked like more mature cells) when cells were plated at 1 to 1000 cells/cm^2[41].

Several factors might be implicated in MSCs aging; their status, source, and pathological process. As a result, the observed variability in senescence in *vivo* and in *vitro* could have been substantially linked to the extracellular vesicles they produced[42].

Senescence-associated changes in the MSCs could explain the observed microvesicles in the present study. The accumulated end product of metabolism, lipofuscin and /or pathological process stimulated their senescence in *vivo*.

In accordance with these suggestions, the characteristics of MSC-derived microvesicles (MSC-MVs) have been studied before by author who detected evidence that these vesicles were numerous in a later passage (LP) in comparison to those in an early passage (EP) that might reflect the senescence in their parental MSCs. Thus, MSC-MVs might have been considered a key factor in the senescence-associated secretory phenotype of MSCs, and/or "potential biomarker" for monitoring MSCs senescence[43].

Exosomes originated from endosomes varied from 40 to 100 nm in diameter. They were released from multivesicular bodies by exocytosis. MVs looked like exosomes, with a larger size, within the limit of resolution of the light microscope vary from 50 to 1000 nm in diameter released from the cell membrane budding[44].

The observed vesicular cytoplasm in MSCs in the control aged group might be explained by the aging-induced degenerative changes, owing to free radicals these changes improved after resveratrol treatment in a duration-dependent manner that confirmed the antioxidant/ free radicles scavenger role of resveratrol, furthermore autophagy might be responsible for the disappearance of those vesicles after resveratrol treatment. Authors confirmed previous suggestions and mentioned resveratrol as a ROS scavenger and iron chelator and inducer of autophagy and mitophagy[45].

A progressive increase in spindle-shaped and branched cells was observed after resveratrol treatment compared to those of the control group. Syncytial like arrangement was observed after resveratrol treatment. With time, confluent and highly expanding closely opposed fibroblast-like spindle-shaped cells were prominent. Some cells were characterized by anastomosing cellular processes, progressive reduction in heterochromatin in nuclei associated with euchromatin increase, as well as finely stippled granulated filamentous cytoplasm.

In addition, some authors described senescence-associated heterochromatic foci (SAHF) as multinucleated heterochromatin foci that caused morphological alterations in senescent cells within the culture[46,47].

The observed condensed and/or heterochromatic nuclei observed in the control aged group with a progressive increase in euchromatin and reduced heterochromatin after "resveratrol therapy" might be explained in a duration-based manner resveratrol capability to reverse physiological aging changes, especially chromatin organization.

It has been discovered that relocating muscle stem cells and neural stem cells to a young systemic environment could restore their regeneration capability in old muscle and nerve tissues[48,49].

Other authors added that aged stem cells in their milieu were susceptible to reactive oxygen species, harmful chemical agents, or physical stresses that triggered premature senescence and accelerated cell death or cellular transformation[50].

The loss of "stem cell frequency" with aging has evoked debate, some studies suggested a decline in MSC number of older individuals[51,52,53,54]. At the same time, other scientists did not find any significant changes[55,56]. This could be due to different volumes of BM aspirate used as well as various processing methodologies (e.g., direct plating vs. density centrifugation[51,52,57,58]

Authors examined the effect of aging on the proliferative and colonogenic potential of "cultured MSCs" by colony-forming unit-fibroblast (CFU-F) assay and found that senescent MSCs exhibited a decreased level of CFU-F[20].

Researchers are in agreement with our suggestions and quantified cell yield and viability to assess expansion properties, proliferation, senescence, and colony formation. They confirmed aging effects on bone marrow-derived MSCs in the form of slow proliferation rate, growth state, colonogenic capacity, and exhibiting senescent phenotype[59]

In the present study, RSV significantly improved cell colony surface area, colony-forming unit assay (CFU), and cell viability (MTT) after two weeks resveratrol therapy. However, significant result regarding the shortening of population duplication time was obtained after only one week of resveratrol ingestion.

Several authors were in accordance with our findings and observed that aging BM-MSCS exhibited low MTT absorbance (cell proliferation assay) that reflected impaired proliferation and viability correlated with age progress[60].

Meanwhile, others reported that MSC senescence was inhibited by resveratrol because it increased MSC proliferation. Furthermore, it promoted osteogenic differentiation, resulting in bone production and the prevention of bone loss[61].

Moreover, others studied the neuroprotective effect of resveratrol and postulated promising positive results by confirming improved cell viability and mitochondrial integrity as demonstrated by increased MTT absorbance capacity. It has been found that resveratrol improved cell viability MTT assay under oxidative cell stress compared to the control group[62]

Meanwhile, others characterized resveratrol as an active redox molecule and discovered that it had lethal effects on several cell types, including fibroblast cell lines and tumor human cells, at higher concentrations. Resveratrol improving effects of all senescence-induced MSCs changes might be referred to its antioxidant and or/ free radicals scavenger effects, thus alleviating the negative aging impact on the MScs microenvironment and increasing their proliferation potential[63,64].

Furthermore, it has been found that antioxidants might be used for the delay and/or prevention of chemotherapeutic drug-induced enhanced reactive oxygen species (ROS), loss of telomeric protective activities, DNA damage, and oncogene activation[65,66]. Thus , prevention of senescence and its concomitant oxidative stress might be exerted by antioxidants treatment[67,68].

In the present study, regarding surface marker expression, significant positive CD90 was demonstrated after two weeks and four weeks of resveratrol treatment compared to that of control group. A Significant increase in the negatively expressed CD31 after two weeks and four weeks resveratrol treatment. CD34 marker was negatively expressed in all groups.

A similar observation was reported in adipose-derived human MSCs, in the form of reduced expression of some positive markers (CD 105, CD73, and CD 90) associated with advanced passages, P5, P8 respectively[18].

Interestingly, the authors speculated that immunophenotype surface marker expression could be used to distinguish between distinct manifestations of MSC senescence. Others observed that surface marker expression was affected by the advancement of in *vitro* cultivation. In contrast to advanced passages, immunophenotyping was highly displayed in early passages[69].

Moreover, considering cell cycle and DNA monitoring, we observed that resveratrol treatment improved aneuploidy proportion significantly in duration dependent manner. Full euploid cells were obtained after four weeks of resveratrol treatment. We assumed that resveratrol could decrease age-associated DNA damage and repair occasional breaks successfully. This explained the positive proliferative effect and perfect cell cycle progression.

These suggestions were in accordance with some authors who explained aging changes in all eukaryotes, including humans, in the form of "chromatin organization" and "gene expression" patterns. Alterations in "chromatin organization" may underpin age-related changes in gene expression and enhance the aging process[70,71].

It has been found that age-related reduction in organ and tissue function cells might be explained by substantial transcriptional alterations[72]. Meanwhile others reported a major transformation in gene expression was association with aging in variable tissues[73,74,75]. Moreover, tumorigenic growth factors might be secreted during aging MSCs replication in *vitro* in concomitant with chromatin changes in cellular culture[76]. Similar chromatin modifications were seen in typically aged tissues, contributing to normal human aging[77,78].

Physiological aging might be explained by reactive oxygen species (ROS) as a "byproduct" of mitochondrial respiration and the ensuing DNA damage, as well as ionizing radiation[79].

We used BubR1 immunoantibodies to get evidence of resveratrol impact on BubR1 sequential expression in the present study. We observed that resveratrol elicited a positive effect on BubR1 expression, and significant morphometric results were obtained after two weeks of treatment for the other group.

The observed significant increase in anti BubR1 antibody positively reacted cells in comparison to control, one week and two weeks resveratrol treated groups also associated with significant progressive reduction of the percentage of aneuploid cells down to zero after four weeks of resveratrol treatment. These findings might confirm the natural activating effect of resveratrol on SIRT1, that might have a great impact in upregulation of BubR1 expression in the present work, the latter was thought to be both a "regulator" of the normal aging process and a key regulator of the mitotic spindle assembly checkpoint, ensured equal chromosome distribution during mitosis.

BubR1 was first described as an inhibitor of the anaphase-promoting complex (APC/C), an E3 ligase complex that promoted the shift from metaphase to anaphase by degrading cell cycle regulators[80].

BubR1 exerted its "prophylactic impact" by correcting "mitotic checkpoint" impairment and microtubule-kinetochore attachment defects. Furthermore, sustained high expression of BubR1 extended lifespan and delayed age-related deterioration and aneuploidy in several tissues. Collectively, these data uncovered a generalized function for BubR1 in counteracting defects that caused whole chromosome instability and suggested that modulating BubR1 could provide a unique opportunity to extend a healthy lifespan[81].

Moreover, BubR1 might be considered as "a director" of the normal aging process by ensuring perfect "chromosome segregation" during mitosis and considered the primary mechanism to ensure that the two daughter cells received the same amount of DNA. Meanwhile, it has been found that failure of this mitotic spindle assembly checkpoint could result in "aneuploidy,"; a state of having abnormal numbers of chromosomes[82].

Sirtuin 1 (SIRT 1) is a nicotinamide adenine dinucleotide (NAD+)-dependent protein deacetylase and master metabolic regulator linked to several disorders associated with aging. SIRT1 activation, primarily mediated by resveratrol, may play a role in cellular energy status, redox reaction levels, longevity, and lifespan[83].

NAD+ levels in the cell were an important sign of cellular energy status in "redox reactions" because NAD+ could easily convert from the electron-accepting form (oxidizing) NAD+ to the electron-donating format (reducing) NADH and vice versa[84].

The last changes might be explained by SIRTs' numerous cellular "signaling pathways" as anti-inflammation, senescence, apoptosis, DNA damage repair, autophagy, and regulate metabolism in response to cellular energy and redox status[61].

Resveratrol's "biological role," such as "anti-oxidation," "anti-inflammation," "anti-diabetes," "anti-cancer," "anti-aging," "cardioprotection," "chemoprevention," and "neuroprotection," may be exerted through its inherent SIRT1 activation potential[85].

Moreover, it has been found that SIRT 1 is involved in autophagy regulation, Reactive oxygen species modulation, and apoptosis control by increasing "p62 transcription". Expression of SIRT 1 could activate insulin-like growth factor receptor (IGFR) and the downstream kinases; pyruvate dehydrogenase kinase 1 (PDK1), SIRT 1 thus regarded as anti-apoptotic protein[86,87].

It was discovered that resveratrol improved "survival," "insulin sensitivity," "organ pathology," "Peroxisome

Proliferator-Activated Receptor Gamma Coactivator 1-Alpha (PGC1a) activity," and "mitochondrial number" through a mechanism similar to that of calorie restriction[27].

Neverthless, other authors suggested that resveratrol promoted the adenosine monophosphate kinase (AMPK) pathway by direct inhibition of cAMP-degrading phosphodiesterases rather than "directly targeting SIRT1[28].

"AMPK pathway" has been explained by an alternative mechanism of resveratrol action as a calorie restriction mimetic. This is based on "competitive inhibition" of cAMP-degrading phosphodiesterases, leading to elevated cAMP levels. This increased intracellular "Ca2+ levels" and activated the CamKKb- via phospholipase C and the ryanodine receptor Ca2+-release channel. Consequently, resveratrol increased NAD+ and the activity of Sirt1[88].

Many investigations supported similar claims, stating that the effect of resveratrol was dose-dependent. It activated AMPK by lowering energy and increasing the AMP/ATP or ADP/ATP ratios at "high concentrations."[89].

However, others assured that resveratrol could activate AMPK at a concentration of less than ten micromoles per liter[90]. Thus low concentrations of resveratrol (%50 mM), could activate AMPK without decreasing energy[91].

It has been found that resveratrol lowered ATP in a cell line at high concentrations (100–300 mM), producing a mutant subunit of AMPK that rendered AMPK hypersensitive to AMP, resveratrol did not activate AMPK[92].

Accordingly, high concentrations of resveratrol may have a negative impact on the growth of hMSCs and maybe other normal cell types in the body. They concluded that people should be cautious while taking resveratrol supplements daily and that lower daily doses are desirable[93].

CONCLUSION AND RECOMMENDATIONS

Resveratrol exerted anti - aging effect on BM-MSCS in duration dependent manner observed by detection of microvescicles in resveratrol treated groups, boosting growth and proliferation rate, improvement of all aging profiles; CFU, CSA, MTT,PDT improvement of genetic changes and reverse aneuploidy changes also significant stemness marker expression.

We recommend that: low dose of resveratrol might be useful as a protective and /or therapeutic agent from aging changes in future clinical studies.

Hopefully, our results might have positive impact on the scope of stem cell based therapy and regenerative medicine

Future directions

We recommend study effect of resveratrol using animals in pre-aging period trying to find the most convenient age to start it as anti-aging agent and apply it in our daily life.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

تاثير الريسفراترول المضاد للشيخوخة على الخلايا الجذعية الوسيطة المشتقة من نخاع العظام وذلك اعتمادا على مدة تناوله

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مقدمة البحث: الشيخوخة هي حدث طبيعي في الحياة يرتبط بخلل تدريجى فى النظام الخلوي والبيولوجي. وهى عملية تراكمية تنتهي بتدهور عام فى وظائف النظام البيولوجي. الخلايا الجذعية هي خلايا فريدة متنوعة ذات صفات خاصة تؤهلها لتكون وثيقة الصلة باستخدامها فى الطب التجديدى.

الريسفراترول وهو فلافونويد بوليفينول فيتواليكسين تم استخلاصه من احد النباتات والذى عرف باستخداماته فى الطب الصيني التقليدي على نطاق واسع

المواد والطرق البحثية: لقد تم اجراء الدراسة على الخلايا الجذعية المشتقة من نخاع عظام جرذان عمرها ١٥ شهرًا. تم استخدام عدد اربعون من اناث الجرذان البيض و تم تقسيمها إلى أربع مجموعات متساوية تضمن كل منها عشر جرذان؛ (أ) الضابطة ، (ب) المجموعة المعالجة بريسفيراترول لمدة أسبوع واحد ، (ج) المجموعة المعالجة بريسفراترول لمدة اسبوعين ، و (د) المجموعة المعالجة بريسفراترول لمدة أربعة أسابيع .

تم تتبع التغيرات المورفولوجية ، وقياس المؤشرات المعبرة عن مساحةالسطح ، واستخدمت معاييرلقياس نمو وانتشار الخلايا، وحيويتها وقدرتها على تكوين المستعمرات الخلوية وقياس المؤشرات الخاصة بالتدفق الخلوي المناعي ، ومؤشر الحمض النووي, وتقنية الكيمياء المناعية للخلايا وقياس البروتين المضاد للتبرعم وهو احد بروتينات الهيمنة على الانقسام الخلوى الميتوزى.

النتائج: لقد توصلنا إلى نتائج ملحوظة وذات دلالة في تحسين كافة المعايير الخاصة بتتبع نمو وانتشار الخلايا وحيويتها واستقرارها الجينى ودلالات الخلايا الجذعية الوسيطة وظهور البروتين المضاد للتبرعم.

الملخص: اثبت الريسفراترول ان لديه تاثير ايجابى على الخلايا الجذعية الوسيطة المشتقة من الجرذان المتقدمة ف العمر فقد اثبت قدرته على تحسين نمو وانتشار الخلايا وحيويتها والحفاظ على صفاتها الجينية وجميع المؤشرات الخاصة بقياس شيخوخة الخلايا معتمدا على مدة تناوله.