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Osteogenic / Adipogenic Differentiation Of Intact And Ovariectomized Young And Adult Female Rat Bone Marrow Mesenchymal Stem Cells (BMMSC).

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ABSTRACT

Although women both young and old routinely undergo ovariectomy (OVX), little is known about the effect of age related gonadectomy on the osteogenic/adipogenic differentiation of bone marrow mesenchymal stem cells (BMMSC). The viability of 80% confluent BMMBC of adult OVX was significantly lower ($P<0.05$) than other groups. In adult Sham- OVX group, results revealed that the proliferation of BMMSC was significantly higher ($P<0.05$) than other OVX groups. These results indicated that gonads and age might share in the control BMMSC proliferation. Lipid accumulation and total lipid concentration in cells of young and adult OVX groups were significantly higher ($P<0.01$) than that in young and adult Sham- OVX groups. *In vitro* osteogenic differentiation of BMMSC indicated that alkaline phosphatase (ALP) and calcium deposition were significantly higher ($P<0.05$) in cells of adult Sham- OVX group than that of other OVX groups. These results indicated that gonads not only promoted osteogenic differentiation, but also inhibited adipogenic differentiation. In total, these observations underscore the importance of understanding the differential effects of age and absence of gonads on bone metabolism. This step will also provide evidence for the proper collection, handling and isolation of BMMSC to be used as experimental trials in renewing medicine.

Keywords: Adipogenesis, BMMSC, Photomicrography, Osteogenesis.

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INTRODUCTION

Osteoblast, osteoclast and adipocyte are the cellular components of bone that regulate bone remodeling through the life of primates. These cells originated from stem cells and migrated during fetal life to resident the bone marrow. There are two types of stem cells, hematopoietic that regulates osteoclast and mesenchymal which produces osteoblast and adipocyte. The mechanism of direction of mesenchymal cells (MSC) to either type is differing. Osteoclasts are cells responsible for bone resorption **[1]**. These cells share MSC in some morphological and functional properties. Besides remodeling of bone, MSC also responsible for bone destruction associated with inflammatory disease **[2]** .MSC are non-hematopoietic cells characterized by self-renewal and multi-lineage differentiation capacity and have been extensively used in the cellular therapeutic field **[3]**.MSC are the most interesting cells to date, because of their diverse functions. As well as, they have multi-fascinating properties in tissues and organs regeneration, which implies that MSC are capable to direct toward injured tissue, to undergo differentiation, immune medulation and to stimulate endothelial cells for angiogenesis and neovascularization **[4]**.

Disturbance in balance of differentiation may lead to bone tissue loss in the form of disease like osteoporosis. At present, osteoporosis treatment mainly targets the symptoms and complications. Therapeutic mechanisms include acceleration of bone formation, inhibition of bone resorption, and agents that promote bone mineralization. Correcting abnormal bone turnover can improve bone mass and reduce the chance of fractures. However, pharmacotherapy can be prohibitive in cost, take a long time to be effective, and lead to adverse reactions. Prolonged treatment of hormone replacement therapy may elevate the risk of many diseases such as breast cancer, stroke, and cerebral infarction **[5]**. Additionally, Bisphosphonate therapy may lead to extensive suppression of bone formation and even necrosis of the mandibular bone**[6]**. Therefore, it is essential to understand possible risk factors associate with osteoporosis medications. Different literatures were discussed the predisposing factors that affected osteoporosis including the fate of resident MSC in bone marrow. It well known that these cells have the ability to differentiate to either osteogenic or adipogenic pathway **[7]**. However, it is not yet clears surely, how osteogenesis or adipogenesis can be optimized and differentiated. Therefore, the field of osteogenesis or adipogenesis and both external and/or internal bone tissue regeneration are faced pronounced work and study to develop new tissue regeneration approaches to produce bone tissue at the expense of adipocyte for clinical applications.

Harvesting and culture of bone marrow cells from ovariectomized and Sham rats for *in vitro* measuring of osteoblastic and adipogenic differentiation potential of BMMSC was previously done by Gao and coworkers **[8]** without considering effect of age of rats. Therefore, the present work was designed to evaluate the *in vitro* proliferation, differentiation of BMMSC to either osteoblast or adipocyte. BMMSC were obtained from tibias and femur of ovariectomized young and adult female rats compared with Sham- operated. These cells were isolated from mononuclear cells and cultured in appropriate media for calculated days needed to reach 80% confluence. Proliferation of stem cells was detected by formazan assay and examined microscopically and quantitates the ability for differentiation. Alizarin red staining, calcium deposition and alkaline phosphatase (ALP) activity for osteogenesis and oil red - O staining and total lipids assay for adipogenesis.

MATERIALS AND METHODS

All procedures were done based on guidelines of Health Guide for the Care and Use of Laboratory Animals. The ovariectomy was performed under ether anesthesia and all steps were done to minimize animal suffering. Two ages of sixty female Wistar albino rats were used. Adult of 70-80 days and young of 21-28 days were selected. The rats were housed in groups of four to five in the animal room, and were fed rodent ration and were given water ad libitum. For ovariectomy, bilateral operation was performed by dorsal approach **[9]**. The incision was closed with interrupted sutures. Ten control rats were exposed to Sham- surgeries in which ovaries were exteriorized. After surgery, all rats were housed separately and supplied with sufficient food and water available ad libitum.

The rats were divided into four groups, fifteen rats each: 1- Young female Sham-operated group (Young Sham- OVX); 2- Young ovariectomized group (Young OVX); 3- Adult female Sham - operated group (Adult Sham- OVX); 4- Adult ovariectomized group (Adult OVX). Four weeks' post-surgery, rats were euthanized.

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The bone marrow (BM) was harvested by introducing alpha minimum essential medium (α-MEM, Invitrogen, USA) in the tibia and femur of each rat in OVX and Sham - operated animals after osteotomy at the femur and tibia heads to obtain the contents. The BM of each animal was diluted with α-MEM and stained with Giemsa stain after methanol fixation (Stock solution was prepared by dissolving 0.3 gm of Giemsa powder (Sigma-Aldrich) plus 25.0 ml glycerine and 25.0 ml absolute methyl alcohol. Working solution was prepared by mixing one part of the stock solution with nine parts of phosphate buffered saline solution (PBS), PH 7.2). This step was done to know cellular compartments and status of bone marrow (Figure I).

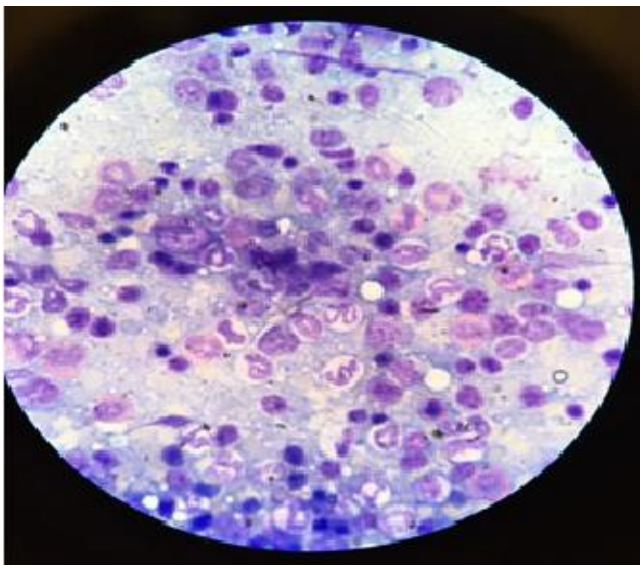

Fig 1: Cellular elements of rat bone marrow aspirates stained with Giemsa.

Isolation of BMMSC from each harvested BM was performed using either equal volume of Ficoll-Histopaque®-1077 (Sigma-Aldrich) or same volume of sodium carbonate buffer solution (0.1%, pH= 7.3) for lyses of erythrocytes **[10]**. Particularly, 1ml volume of BM was used on the same volume of Ficoll or buffer solution for separation of mononuclear cells (MNC) at low speed centrifugation for 20 minutes. The MNC was aspirated and then mixed with α-MEM to calculate viability %. Appropriate suspension of all nucleated cells was seeded after counting at a density of 1 × 106 into culture dishes (Figure 2). The non-viable blue and viable transparent cells were quantified in a Neubauer chamber haemocytometer as follow:

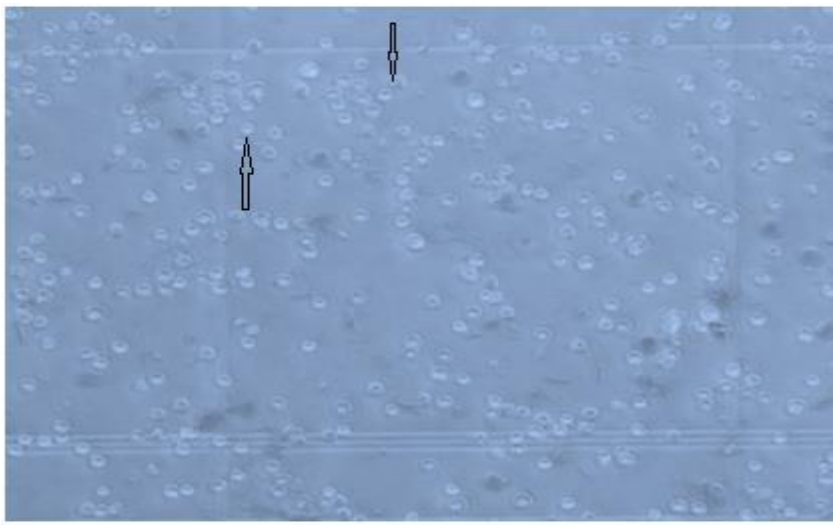

Fig 2: Viability of MNC (arrow) by trypan blue stain (light microscope, X40).

Total count= number of cells (16 square) X dilutionX104= count X2X105 /1ml. The viability % = 100 X number of viable cells /number of total cells (viable + dead).

Cells were incubated to adhere at 37°C and 5% CO2 overnight and non-adherent cells will be washed out with medium changes. The attached cells were maintained in α-MEM supplemented with fetal bovine serum (FBS, 20%), L-glutamine (2 mM), 2- mercaptoethanol (55 µM), and two types of antibiotics. After that, the medium was changed twice weekly. Upon reaching approximately 80% confluence, detachment of adherent cells was performed using trypsin- EDTA (0.25%) and washed twice with PBS, then centrifuged at 2000 rpm for 15 minutes, and suspended under the same culture conditions. The cells were picked and elected as passage 1(P1), and serial passage numbers of 0.25 x 106 cells will be designated thereafter. For assurance of BMMSC isolation, 0.2 × 106 cells after washing with PBS containing 1% bovine serum albumin (Sigma-Aldrich) were stained with anti-CD34 and anti-CD45 antibodies (BD Biosciences). Stained cells and isotype control were analyzed by a FACS Calibur flow cytometer (BD Biosciences). Analysis showed that cultured cells failed to express CD34 and CD45 (data not showed). The cells were picked and elected as passage 1(P1), and serial passage numbers of 0.25 x 106 cells will be designated thereafter. All materials used were purchased from Sigma-Aldrich. Before culturing in osteogenic or adipogenic media, the BMMSC of each animal of all groups were proved for viability by using trypan blue stain. The cells were suspended at appropriate density of 20.000 cells/cm2 in complete culture media inside sterile culture plates. The plates were incubated at 37°C in a humidified atmosphere of 5% CO2.The medium was changed regularly twice a week.

Sub culturing (Passaging) was performed when adherent cells of first cell culture reached 80% confluence and it named passage zero. The following passages were named consequently. Adherent cells of primary cell culture were released by sterile trypsin solution at 37°C. FBS (100µl) was added to inactivate the trypsin. The cells were pelleted after centrifugation at low speed followed by division into two parts by the same media. Each part was plated (passaging) to increase cell number. All the previous procedures were directed under aseptic conditions in an air filtered laminar flow safety cabinet and by using sterile instruments.

Estimation of the proliferation capability of stem cells was measured in both primary cultures and subcultures. The proliferation capacity was assayed by determine the days of the ability to form 80% confluence. Cultures were monitored using inverted light microscope (Olympus, USA) with digital camera for capturing images. Furthermore, the cells were tested for the ability to form colonies and dimethylthiazol-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) assay were performed to compare the proliferation of the cells.

To measure the ability of stem cells for the capacity to form colony, a suspension of 100 cell/ml was cultured in a 3.5 cm dish in complete culture media and the cultures were observed under inverted light microscope after three weeks. Aggregates with more than 50 cells were counted as colonies.

Indirect MTT assay to determine the activity of mitochondrial enzymes was done. Cells were incubated with 0.2 mg MTT /ml appropriate media, for one hour at 37°C in 96-well plates to form formazan after reduction. The solution was then removed and 0.04 N HCL in 1ml isopropanol was added to solubilize formazan. The quantity of formazan after shaking 5 min then assayed calorimetrically at a wavelength of 570 nm **[11]**.

For adipogenic differentiation induction, fifth passage cells derived from each rat were maintained with adipogenic differentiation media for 3 weeks. This media must contain factors essentials for adipogenesis as glucose (4.5 g/L), dexamethasone (1 µM), indomethacin (0.5 mM), 3-isobutyl-1- methylxanthine (0.5 mM) and insulin. The percent of values was conducted as previously described [10].

Santosh and coworkers **[12]** mentioned how to evaluate the adipogenic differentiation of BMMSC. Oil red- O stock solution (0.5%) in isopropanol was prepared and filtered with 0.2 mm filter. Before staining, working solution was prepared from a mixture of 12 ml stock solution and 8 ml distilled water and left for 1 hr. at room temperature, and then it was filtered prior to use. Conflated cells were fixed in 96-well plates with paraformaldehyde (4%) in PBS for 20 min. Fixed cells were stained for 20 min and then rinsed three times with PBS. Cells were examined by inverted microscope. The dye inside the cells was then eluted by incubation with isopropanol for 15 min. At the end, 200 µl of the solution was pipetted into each well of a 96-well plate, (Fig.3) and the optical density at 540 nm was read using ELISA plate reader (MR 700 Microplate Reader, Dynatech Laboratories Inc. Product, USA) **[12]**.

Chloroform methanol solvents (2:1, v/v) extraction method was used for total lipids assay according to Petkovic´ and colleagues **[13]**. Briefly, two ml from solvent was added to cell pellets obtained by centrifugation at low speed. The mixture was agitated and incubated at room temperature for 20 min. Addition of 0.9% NaCl (400 µl) to the mixture followed by agitation and centrifugation. The lower layer was collected for the analysis by using sulfo-phospho-vanillin calorimetric kits (Sigma-Aldrich).

The cultural condition of osteogenic differentiation was used as established **[14, 15].**To provide osteogenic conditions, the culture medium of the confluent cultures of passage 3 derived from each rat was substituted with osteogenic medium consisting of α-MEM with FBS (10%), penicillin-streptomycin and glutamax supplemented with osteogenic medium consisting of L-ascorbic acid 2-phosphate (100 µM), L-glutamine (2 mM), dexamethasone (10 nM) and β-glycerophosphate (2 mM). All materials used were purchased from Sigma-Aldrich. The differentiation cultures were maintained for 3 weeks. The media were changed every two days.

Alizarin red stain was used to evaluate the presence of calcified tissues and mineralization potentiality inside MSC. After rinsing cells with PBS, 10% buffered formalin was used to fix cells for 10 minutes. Rinsing of formalin was done using distilled water, followed by staining with freshly prepared Alizarin red solution (1%) for 20 min. The stain was removed by washing with distilled water. Finally, keeping cells wet by one ml of distilled water was done. Detecting of mineralization of all groups was performed during 3 weeks while the cells were maintained in the differentiation media and at the end. The mineralized nodules were showed and graphed using inverted microscope **[16, 17].**

Alkaline phosphatase (ALP) activity that indicates osteogenic differentiation was performed using a method of Choi and coworkers **[18]** and Leskelä and colleagues **[19]**. Briefly, MSC were cultured in osteogenic medium for three weeks, and then rinsed with PBS and trypsinzed for elution. Cell lysis was done by freezing and thawing for 2-3 times. 100 µL/well of the ALP assay kit (Abcam) was then added to produce p-nitrophenol from p-nitrophenyl phosphate. P-nitrophenol was then assayed at 405 nm and the results were expressed as units/mg protein which was determined by method of Lowry **[20]**.

The differentiation of BMMSC to bone tissue was indicated by the amount of calcium deposited as previously described **[21]**. Briefly, fixed number of colonies from plate showing 80 % confluence were washed twice with PBS and hydrochloric acid extracting solution were used (0.5 N). Calcium was extracted from cells by agitation for 5 hr. at 4◦C, followed by centrifugation at low speed. The supernatant was used for assay calcium by calorimetric assay kit (MAK022- Sigma-Aldrich). Total calcium was calculated after measuring the absorbance at 575 nm. Standard solutions were prepared in parallel to express the reading as mg/well.

Program software of SPSS version 19 was used and both ANOVA and Tukey's post-test were done. Data were expressed as the mean ± standard error (SE). Correlation analysis to obtain P values and Pearson coefficient was evaluated.

RESULTS

BMSC of different groups of rats were isolated from bone marrow after adhesion on the bottom of the culture dishes. After one to two days from initial seeding, cells were observed by inverted microscope and it began to form process and some cells became spindle and appeared as fibroblast (Figure 3). Cells from all groups continued to proliferate and propagate until 80% confluence and the dish area became covered with cells (Figure 4). Following confluence, the cells were passed successfully up to the third passage (P 3). Subcultures from either tissue tended to exhibit an accelerated growth, so the cultures reached confluence in shorter time than primary cultures (about 3-4 days). During expansion cultures in P2 and P3, the number of the cells kept increasing. The cells started to compress and gradually lost their spindle- like appearance.

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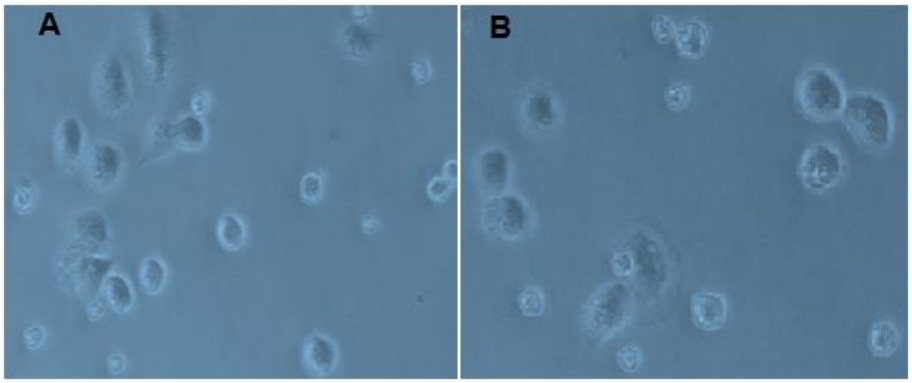

Fig 3: Photomicrograph of culture MNC after 2 days showing morphological diversity and fibroblastic appearance (X20 and X40 for A and B).

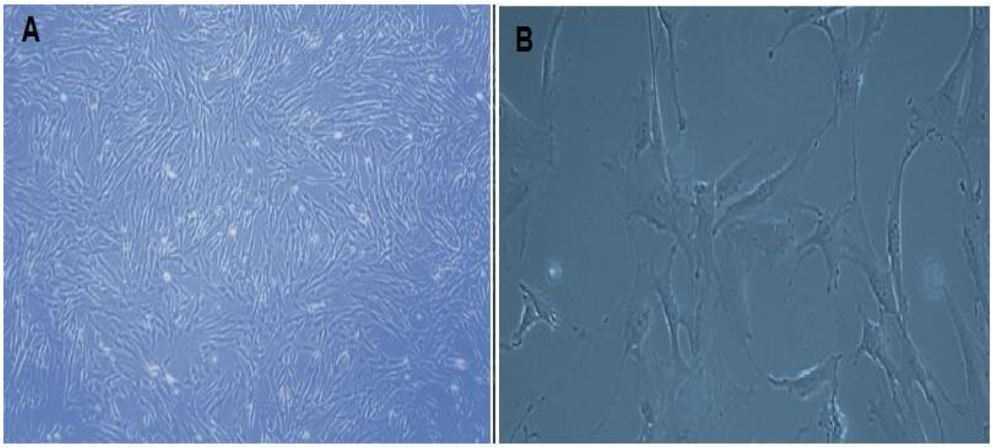

Fig 4: Photomicrograph of culture BMMSC showing spindle shape and stellate shaped appearance. Cells reached 80% confluence between days 15.351 and 22.365 according to groups (X10 and X40 for A and B).

The viability % of MNC and BMMSC upon reaching 80% confluence is shown in table (1). Data revealed that there was no significant variation in the viability % of MNC between all groups of the experiment. However, the viability % of 80% confluent BMMBCs of adult OVX rats was significantly lower ($P<0.05$) than that of young Sham- OVX, young OVX and adult Sham- OVX groups. Time of BMMSC isolated from different groups of rats to reach 80% confluences shown in table (1). Data revealed that there was no significant variation in the days to reach 80% confluence in all groups examined. The proliferation as expressed by OD of 80% confluence of stem cells detected by formazan assay was shown in table (1). In adult Sham- OVX group, results revealed that the proliferation of 80% confluent stem cells was significantly higher ($P<0.05$) than other OVX groups.

Table (1): Viability of MNCs and BMMSC (%), Time of BMMSC to reach 80% confluence (days) and proliferation detected by formazan assay (OD) of different groups of rats

Groups	MNCs viability %	BMMSC viability %	Time to reach 80% confluence (days)	Formazan OD (A=570 nm)
Young Sham- OVX	96.22±1.24	93.18±2.42	16.312±2.111	0.134±0.035
Young OVX	94.54±2.14	85.22±3.19	18.117±3.321	0.092±0.009
Adult Sham- OVX	93.46±2.11	95.24±1.33	15.351±1.180	0.211±0.015*
Adult OVX	97.37±4.24	81.24±1.36*	17.516±3.421	0.093±0.028

Data are represented as the mean± standard error (n=4).

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Asterisks * indicate significant at P<0.05 compared with non-marked groups of the same column. OVX (Ovariectomy).

Photomicrograph of *in vitro* adipogenic differentiation of BMMSC was shown in figure 5. The differentiated cells of young and adult Sham- OVX groups showed moderate accumulation of oil red- O stained lipid droplets. Young and adult OVX groups showed very high stain. Results revealed that lipid accumulation in young and adult OVX groups was significantly higher (P<0.01) than that in young and adult Sham- OVX groups. (Figure 5).

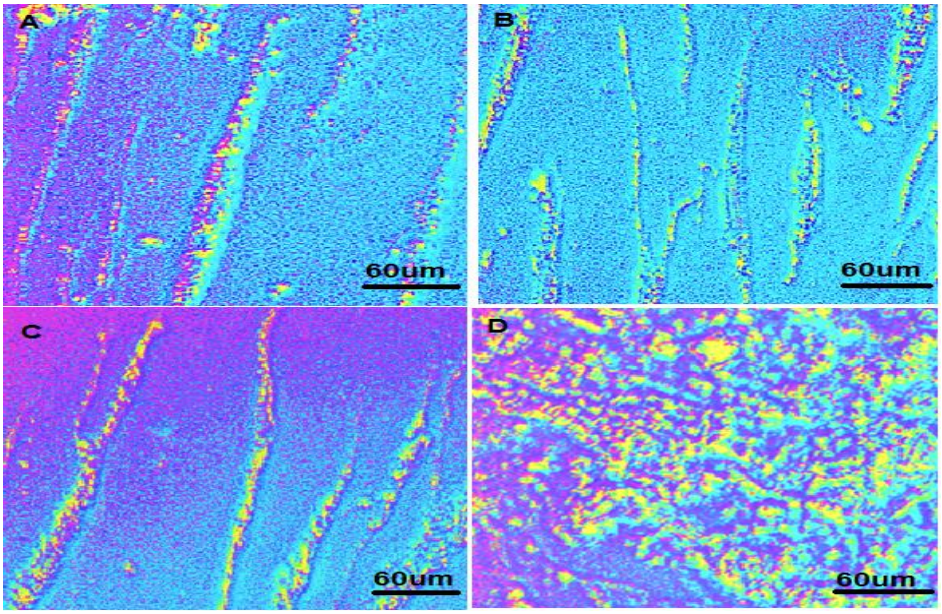

Fig 5: Photomicrograph of *in vitro*adipogenic differentiation of BMMSC. The differentiated cells accumulated oil red- O stained lipid droplets. (A)Young Sham-OVX group showing medium stain. (X40). (B)Young OVX group showing very high stain (X40). (C)Adult Sham-OVX group showing medium stain (X40). (D)Adult OVX group showing very high stain (X40).

Quantification of lipid accumulation by elution of oil red-O from the stained cells was shown in table (2) and figure (6). Total lipid concentration in oil red-O stained cells was shown in table (2). The results showed that total lipid concentration was significantly higher in young OVX (P<0.01) and adult OVX (P<0.05) groups than young and adult Sham- OVX groups.

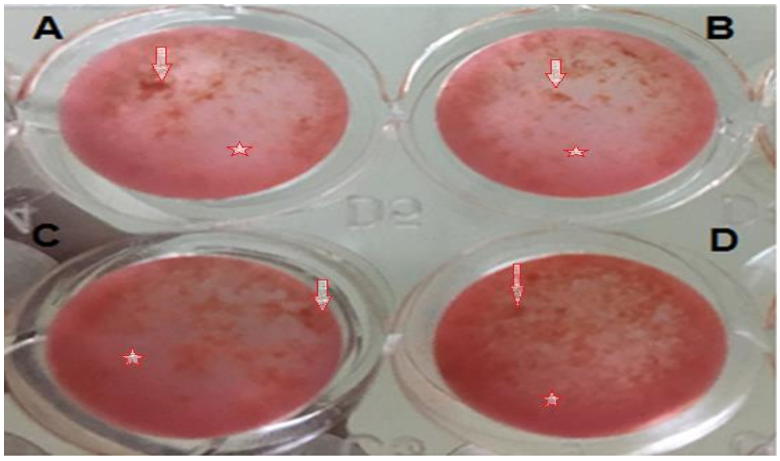

Fig 6: ELISA Plates showing elution of oil red - O stain (star) from the adipogenic differentiated cells (arrow) by incubation with isopropanol for 15 min. (A)Young Sham- OVX showing medium stain, (B) Young OVX

showing very high stain, (C) Adult Sham- OVX showing medium stain, (D) Adult OVX showing very high stain. The OD at 540 nm was read using plate reader.

Table (2): Quantification of lipid accumulation by elution of oil red-O from the stained cells with isopropanol (OD) and total lipids assay (ug/10ul)

Groups	OD (A=540)	Concentration (ug/10ul)
Young Sham- OVX	0. 371±0.024	5.244±1.087
Young OVX	0. 823±0.040**	9.184±1.158**
Adult Sham- OVX	0.292±0.028	4.331±1.525
Adult OVX	0.422±0.011**	7.298±1.234*

Data are represented as the mean± standard error (n=4).
Asterisks * and** indicate significant at P<0.05 and P<0.01respectively compared with non-marked groups of the same age and column. OVX (Ovariectomy).

In vitro osteogenic differentiation of BMMSC in response to Alizarin red staining were shown in figure (7). Scattered nodule like structures dispersed within the culture cells started to appear and were stained red by Alizarin red, an event that was seen in all groups. At day 21, photomicrograph of young OVX & adult OVX and young Sham- OVX groups showed low scattered orange to red calcified nodules in response to Alizarin red staining while photomicrograph of adult Sham- OVX showed medium red calcified nodules.

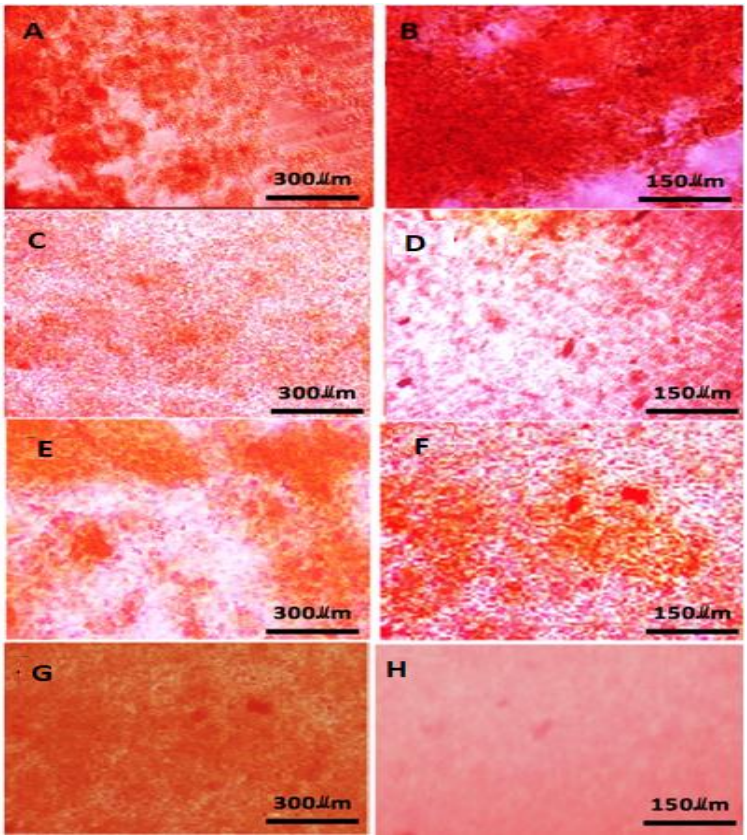

Fig 7: Photomicrograph of *in vitro* osteogenic differentiation of BMMSCs of adult Sham-OVX group (A&B) showing highly scattered red calcified nodules in response to Alizarin red staining (X20& X40). Photomicrograph of young Sham-OVX (X20& X40 for C & D) showing medium scattered red calcified nodules. Young OVX group (X20& X40 for E & F) showing low scattered red calcified nodules. Adult OVX group (X20& X40 for G and H) showing very low scattered red calcified nodules in response to Alizarin red staining.

Alkaline phosphatase (ALP) activity as expressed by OD and concentration (U/mg. protein) were shown in table (3). It was found that OD and activity of ALP were significantly higher (P<0.05) in cells of adult

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Sham- OVX group than that of other OVX groups. Calcium concentrations in the wells were shown in table (3) and figure (8). The results showed that calcium deposition in adult Sham- OVX group was significantly higher ($P<0.05$) than that in other OVX groups.

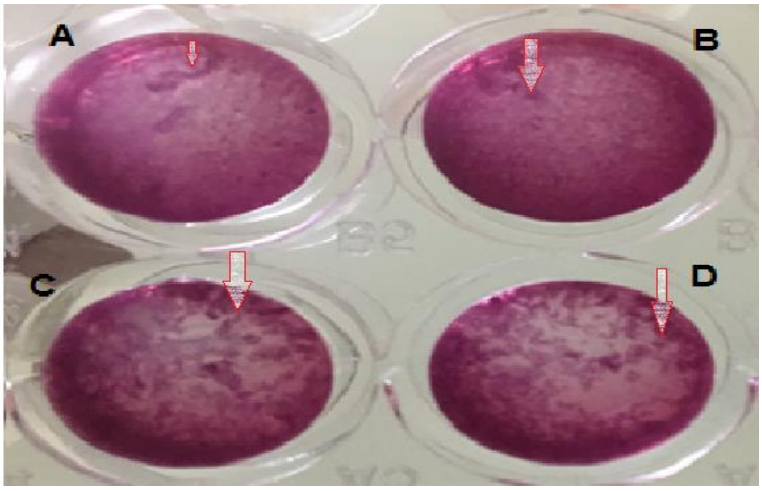

Fig 8: Alizarin red stain (1%) was used to evaluate the presence of calcified tissues and mineralization potentiality (arrow) inside MSC. (A) young OVX showing low scattered red calcified nodules, (B) adult OVX showing low scattered red calcified nodules, (C) young Sham- OVX showing medium scattered red calcified nodules, (D) adult Sham- OVX showing medium scattered red calcified nodules. The OD was read at 540 nm using ELISA plate reader.

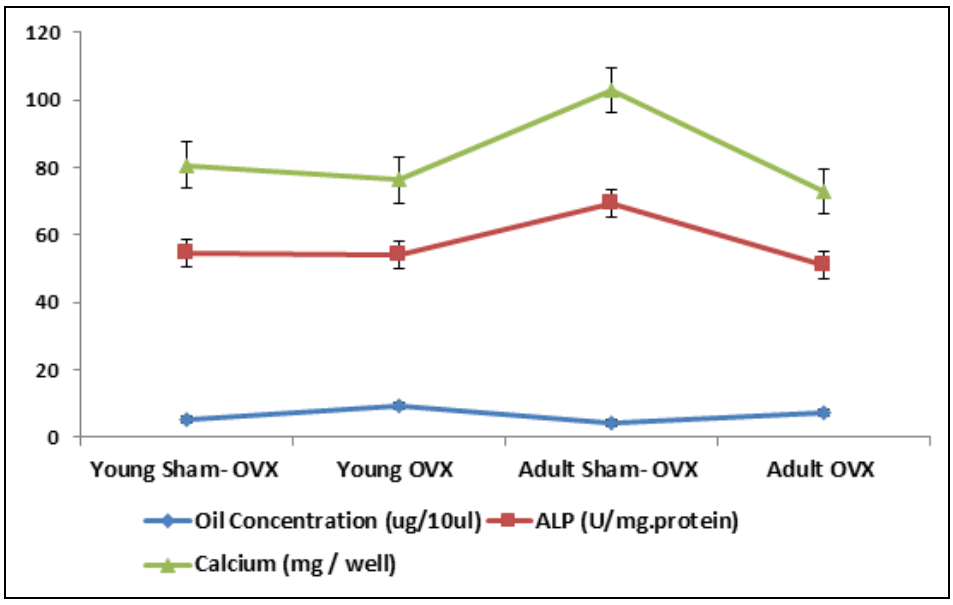

Fig 9: Correlation of calcium, ALP and oil concentration of BMMSC isolated from different groups studied. Calcium and ALP concentrations were showed a significant Pearson correlation to oil concentrations. It was recorded that Pearson correlation of calcium and oil concentration was 0.814 at P-value 0.093 while the correlation of ALP and oil concentration was 0.737 at P-value 0.155. However, Pearson correlation of calcium and ALP was 0.989 at P-value 0.001.

Table (3): Alkaline phosphatase (ALP) activity as expressed by OD (A=405 nm) and concentration (U/mg. protein) and calcium deposition (mg / well)

Groups	ALP (OD)	ALP (U/mg.protein)	Calcium (mg / well)
Young Sham- OVX	0.124±0.02	49.23±3.35	26.19±1.27
Young OVX	0.090±0.01	44.66±5.25	22.32±2.51

Adult Sham- OVX	0.289±0.08*	64.87±6.85*	33.65±3.26*
Adult OVX	0.093±0.02	43.61±3.94	21.85±3.38

Data are represented as the mean± standard error (n=4).
Asterisks * significant at P<0.05 compared with non-marked groups of the same age and column.

DISCUSSION

Due to ethics related to the use of embryonic stem cells (ESCs), laboratory attention was concentrated on stem cells derived from adult tissues. However, BMMSC gather the basic characteristics of stem cells abilities to self-renew and differentiate into multiple cell types. Commonly, there are three important features allowing BMMSC to be isolated from various species are the plastic surfaces adherent ability, the high proliferation capacity, and the greater vulnerability to trypsin digestion compared to other BM cells **[22]**. Therefore, BMMSC was used in the present study.

Bone formation is a physiological process by which old bone is replaced by new one. Old bone was resorbed by osteoclasts while new formation by osteoblasts **[23]**. This balance between these cells helps to maintain bone stability as previously documented **[23]**. Several investigators were referred to another balance of differentiation of MSC to either adipocyte or osteocytes**[7, 24]**. This balance was depending on many factors including age and hormonal level and it might lead to osteoporosis. This work was focused on the effect of age difference besides the presence or absence of gonads in female rats.

Our Data revealed that there was no significant variation in time of BMMBC to reach 80% confluence in all groups examined. In addition, there was no significant variation in the viability percentage of MNC between all groups of the experiment. However, the viability percentage of 80% confluent BMMBC of adult OVX group was significantly lower than that of young and adult Sham- OVX and young OVX groups. Moreover, results revealed that the proliferation of 80% confluent stem cells as detected by formazan assay was significantly higher in adult Sham- group than OVX groups. These results collectively indicated that removal of gonads in both young and adult female rats was associated with the decline in the above-mentioned parameters. This data concluded that the presence of gonads is essential for proliferative function of stem cells and provide insight into the work of stem cells independent on the age of OVX. It was noted previously that women both young and old routinely undergo OVX and this work regarding the presence of gonads is essential for proliferative function of stem cells. Previous study indicated that female sex hormone estrogen influence and control MSC proliferation **[25]**. This effect was characterized in stem cells isolated from bone marrow of female rat. However, different studies on male and female sex hormones beside other hormones can affect stem cells proliferation and differentiations **[26, 27].** Previous studies discussed how estrogen direct the differentiation. Authors suggested that the effect of estrogen on their receptors might affect the function of those cells **[28, 29].** Thus, modulation of the function of stem cells through sex hormones may be the goal of recent therapeutic efficacy **[30, 31].** The molecular mechanism by which steroids enhance proliferation was previously mentioned by activating Notch signaling pathway and up regulation of ERs transcripts activation **[32, 33].** However, the mechanisms of the gender difference of estrogen regulation are still not elucidated.

Regarding the photomicrograph of *in vitro* adipogenic differentiation of BMMSC as indicated by accumulation of oil red- O stained lipid droplets; the intensity of stain was differing between both ages and the orchiectomy. While the medium stain was observed in young and adult Sham- OVX groups, the OVX groups of both ages showed very high stain that indicated pronounced adipogenic differentiation after ovariectomy. This data was matching with previous study of Goa and coworkers **[8]** that revealed bilateral ovariectomy positively influenced the adipogenic differentiation potential of BMMSC, this action may be partly mediated through adipogenic special markers peroxisome proliferators activated receptor gamma, CCAAT/enhancer binding protein alpha and adipocyte lipid-binding protein 2.

Quantification of lipid accumulation by elution of oil red-O from the stained cells revealed that accumulation in young and adult OVX groups were significantly higher than that in young and adult Sham-OVX groups. These results were confirmed the photomicrograph observation. Estrogen has been proven previously to inhibit adipocyte progression into fat and act on osteoblasts or BMMSC of the target site and inhibit the transcript levels of lipoprotein lipase, influencing the distribution of body fat **[34]**. Okazaki and coworkers **[35]** indicated that estrogen not only promoted osteogenic differentiation, but also inhibited adipogenic differentiation.

For osteogenesis, the media used in the present study contain substances considered as stimuli such as ascorbic acid, β-glycerphosphate (β-GP), dexamethasone and Vitamin D3. Ascorbic acid actively induces mineralization and promotes collagen synthesis. β-glycerphosphate is involved in the construction of mineral hydroxyapatite within the collagenous matrix **[36]**. While ascorbic acid induces the activity of ALP, where it splits phosphate from the added β-GP, phosphate move in the cell via Na2+ transporters **[37]**. Photomicrograph of *in vitro* osteogenic differentiation of BMMSC indicated that young and adult OVX groups showed low scattered orange to red calcified nodules while adult Sham- OVX group showed medium nodules than young Sham- OVX group who showed low nodules in response to Alizarin red staining. For further investigate the osteogenic differentiation, ALP activity and calcium concentrations were done. It was found that activity of ALP was significantly higher in cells of adult Sham- OVX group than that of other OVX groups. However, the results showed that calcium deposition in adult Sham- OVX group was significantly higher than OVX groups. This data indicated that OVX in both ages used in this study was accompanied by a decline in osteogenic parameters. These results were disagreeing with the finding of Gao and colleagues **[8]** that may be due to different range of rat age. However, Bergman and coworkers **[38]** indicated that osteogenesis lowered and adipogenic differentiation elevated with aging in animals, and that these are the main causes of senile osteoporosis. Excessive bone resorption leads to lowered bone mass and destruction of the bone structure**[39, 40].** Therefore, according to the pathogenesis of the osteoporosis, a treatment that increases the number of osteoblasts and decreases the number of adipose cells, in theory, will lead to a cure for disease. Several signals, including physical, chemical and biological, can modify MSC differentiation toward fat or bone formation. Once the balance of osteogenesis and adipogenesis is disrupted, osteoporosis may occur. Thus, the balance between osteogenesis and adipogenesis would be a therapeutic agent for treatment of bone loss and fat accumulation.

Scientists reported that the low bone volume resulting from osteoporosis was correlated to increase in marrow adipose tissue. This relation was affected by age and sex. However, if this differentiation is disturbed, it may be conducted to a decrease in bone volume. If the number of adipocyte cells elevated and that of osteoblast cells lowered, osteoporosis can result. Gonadectomy was mentioned by Lean and colleagues **[41]** that it conducted to oxidative stress by decline in antioxidant enzymes and elevation of reactive oxygen species (ROS). The end results were elevation in osteoclast cells and ability of bone to destruct will be easily [41].

Concerning sex hormones, estrogen maintains bone density by suppressing bone resorption and enhance bone formation as reported recently **[42]**. Sex hormones are main cues to maintain this balance. This effect might be through their nuclear receptors **[43]**. Martin and colleagues **[44]** indicated that estrogen has an important effect in regulating differentiation of osteoblasts. In addition, estrogen has also been proven to inhibit adipocyte progression into fat **[34]**. Male rodent showed elevated bone mass during growth than females **[45]** that concluded, osteoblastogenesis was affected sexually dimorphic and influenced by genetic factors. This proved that the balance of bone cellular element was directed to decrease bone formation in postmenopausal women due to decrease estrogen hormone **[46]** resulting in an increased risk of fracture associated with osteopenia **[47]**. The availability of cells to form bone marrow was diminished in estrogen deficient individual **[25, 48].** Age-related decline in the number of MSC in the bone marrow has been observed in rodents and humans **[49].** It has been shown that, estrogen administration to ovariectomised rodent was accompanied by decrease in apoptosis of MSC *in vitro* **[50]**. In *in vitro* studies, osteogenic differentiation of bone marrow MSC was increased when estrogen was added to the media due to increase the expression of bone morphogenetic protein, and significantly increases the deposition of calcium and ALP **[51]**.

In total, these observations underscore the importance of understanding the differential effects of age and presence or absence of female gonads on bone metabolism and physiology figure (9). This step will provide evidence for the proper collection, handling, isolation and identification of MSC from BM to be used as experimental trials in renewing medicine. The desired outcome from this work is to provide MSC-based therapy for tissue defect. In addition, depending on the results of this study, a further investigation on senile animals was needed that can be carried out in the future with human clinical trial.

Authors' Contributions

All authors designed the study and contributed to experiments performance, laboratory work analysis and data interpretation, manuscript preparation. They shared in experiments performance. All authors have read and approved the final manuscript.

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