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**Research Article**

# Effect of adipose derived stem cells on ovariectomised Wistar rats

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## ABSTRACT

**Background:** Various clinical trials are going to determine the efficacy of human Adipose Derived Stem Cells (hADSCs) in the treatment of degenerative diseases including osteoporosis. Stem cell therapy for osteoporosis is aimed at inducing new bone formation by the proliferation and differentiation of bone progenitor cells. The therapeutic potential of hADSCs has to be investigated in animal models of osteoporosis before suggesting it as a therapeutic option.
**Methods:** hADSCs were cultured in the Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4 mM L-glutamine and 110 mg/l sodium pyruvate, 10% Fetal Bovine Serum (FBS), 1% penicillin–streptomycin and non-essential amino acids. For osteogenic differentiation of hADSCs, cells were cultured as above then were exposed to osteogenic induction medium for seven days. Intravenous infusion of osteogenesis induced hADSCs was given to 20 ovariectomised Wistar rats three months after ovariectomy (test group) and 20 ovariectomised rats were kept as controls. Rats were sacrificed 35 days after infusion and tibial cross sections at the level of tibio-fibular joint were stained with H&E & Masson's trichrome. The digital slide images were viewed using Aperio Image Scope software.
**Results:** The results showed that there was new bone formation in the test group, indicated by osteoid formation and osteoblasts. There was significant increase in the cortical thickness in the test group when compared with the control group. There was no significant increase in trabecular volume when compared to the control group.
**Conclusions:** hADSCs after osteogenic induction may have the potential to enhance new bone formation and may be useful in the treatment of osteoporosis.

**Keywords:** Adipose derived stem cells, Female wistar rats, Osteoporosis, New bone formation

## INTRODUCTION

Recent progress in stem cell biology has provided a promising strategy for treatment of multiple degenerative disorders.[1] In particular, adult stem cells have emerged as an important issue due to the potential for use of their ex vivo expanded progenies in cell-based regenerative medicine, tissue engineering and cancer therapy.[2] Adult stem cells participate in replenishment of cells that are lost during regeneration of damaged tissue, as well as in normal tissue development. Mesenchymal Stem Cells (MSCs), which possess unique immunosuppressive and anti-inflammatory properties and a capacity for homing to injured tissues, have been isolated from various tissues, including bone marrow, adipose tissue, hair follicles, spleen, placenta, umbilical cord blood, foetal liver and lung.

Adipose derived stem cells (hADSCs) could constitute a promising source of cells for use in cell-based therapy and tissue engineering.[3] The main benefits of Adipose-derived Stem Cells (ASCs) in therapeutic applications, as compared with bone marrow derived mesenchymal stem cells, are that adipose tissue is readily accessible and relatively abundant, and the stem cell population can be easily harvested by simple methods, such as liposuction.

Aging reduces the number of Mesenchymal Stem Cells (MSCs) that can differentiate into osteoblasts in the bone marrow, which leads to impairment of osteogenesis. However, if mesenchymal stem cells could be directed toward osteogenic differentiation, they could be a viable therapeutic option for bone regeneration.[4] Since MSCs are multipotent and have low immunogenicity, they are considered as potential candidates for a variety of clinical applications including cartilage reconstitution and the treatment of rheumatoid arthritis, acute osteochondral fractures, spinal disc injuries, and inherited diseases like osteogenesis imperfecta.[5-7]

Although there are studies done to assess the therapeutic potential of hADSCs in various degenerative diseases, there is very limited information available regarding the effect of intravenous administration of osteogenesis induced hADSCs on osteoporotic bones. Prior to initiating a clinical trial in post-menopausal osteoporotic women, it is reasonable to recommence the evaluation of treatment in ovariectomised 9-month-old female rats. So the current study is aimed at evaluating the effect of osteogenesis induced hADSCs on ovariectomised rats.

## METHODS

### *Subjects*

Female Wistar rats (6months old) bred in King Fahd Medical Research Center, Jeddah were used for the study. All applicable institutional and national guidelines for the care and use of animals were followed. Experimental osteoporosis was induced in forty two female 6 month old Wistar rats by doing bilateral ovariectomy and sham surgery was done in control rats.[8] The rats were then kept in stainless steel cages at room temperature for 3 months. Sham surgery was done in another group of 6 month old rats to compare the bone changes.

### *Culture and osteogenic differentiation of hADSCS*

The culture and osteogenic differentiation of hADSCs was done in the stem cell laboratory of King Saud University, Riyadh. The hADSCs were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 4 mM L-glutamine and 110 mg/l sodium pyruvate, 10% Fetal Bovine Serum (FBS), 1% penicillin-streptomycin and non-essential amino acids.[9] For osteogenic differentiation of hMSCs, cells were exposed to the osteogenic induction medium (DMEM + 10% FBS, 1% Pen/Strep, 50 µg/ml L-ascorbic acid, 10 mM β-glycerophosphate, 10 nM calcitriol and 10 nM dexamethasone) for seven days.[9]

At the end of three months ovariectomised rats and sham surgery rats were sacrificed by cervical dislocation after giving ether anesthesia.[10] The right tibia along with the fibula was dissected out and was kept in 10% buffered formalin. The bones were decalcified using rapid decalcifier (Shandon). After dehydration and paraffin embedding 5 µm thickness cross sections of tibia were made at the level of proximal tibio-fibular joint. The sections were stained by H&E and studied under the microscope to confirm osteoporotic changes in the ovariectomised group.

After confirming the osteoporotic changes, intravenous infusion of stem cells suspended in saline at the dosage of $2.5x10^6$ cells/kg body weight was given to 20 ovariectomised rats (9 months old weighing 250-300 gm) through the tail vein using a 1 ml syringe.[11] The remaining 20 rats were kept as control group. After 5 weeks all the rats were sacrificed by cervical dislocation under ether anesthesia.[12] The right tibia along with the fibula of both the experimental group and the control group were dissected out and was kept in 10% buffered formalin. The bones were decalcified using rapid decalcifier (Shandon). After dehydration and paraffin embedding, 5 µm thickness cross sections of tibia were made at the level of proximal tibio-fibular joint.[13] The sections were stained with H&E and Masson's trichrome staining. The new bone formation was assessed with H&E staining.[14] The cortical thickness and trabecular volume were measured in Masson's trichrome sections using image analyzer.[15]

### *Measurements*

All stained histological sections were examined using an Olympus BX51 microscope (Olympus, Japan) and photomicrographs were taken by an Olympus DP72 digital camera (Olympus, Japan) attached to it. High-resolution whole-slide digital scans of all Masson's trichrome stained histological sections were created with Aperio Scan Scope scanner (Leica Microsystems, Germany). The digital slide images were viewed using Aperio Image Scope software (Leica Microsystems, Germany) and analyzed using Aperio image analysis algorithms (Leica Microsystems, Germany). On the slide images of the test group and control group, cortical thickness was measured at five randomly chosen points, using the linear measurement tool of Aperio Image Scope software (Leica Microsystems, Germany). The mean cortical thickness of the test group and control group were compared. To measure trabecular volume, the bone marrow cavity was outlined as the area of interest. Aperio color deconvolution (color separation) algorithm (Leica Microsystems, Germany) was then applied so as to select and measure the area of only the blue color of bone trabeculae (as stained by Masson's trichrome) and calculate its area percentage relative to the total area of analysis (area of bone marrow cavity).[16] The results were analysed using SPSS software and Student's t test was used to determine the statistical significance.

## RESULTS

The tibial sections stained with H&E in the test group showed evidence of new bone formation with osteoid and osteoclasts (Figure 1). In the control group, the cortical

bone showed resorption cavities and the endosteal surface was irregular and eroded. There was no evidence of new bone formation.

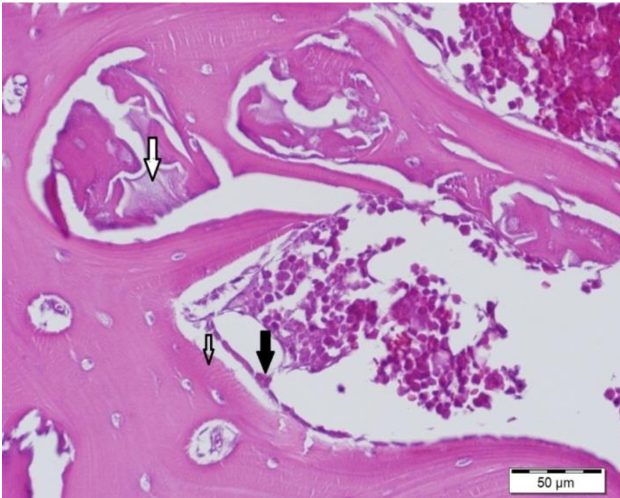


**Figure 1: New bone formation in the test group indicated by osteoid and osteoblasts. Photomicrogrph taken with Olympus BX51 microscope attached with Olympus DP72 digital camera (40X). Black arrow shows the osteoblasts. The white arrows are pointed towards the new bone.**

To measure the cortical thickness, digital slide images were viewed using Aperio Image Scope software. Measurements were taken at 5 randomly selected points and the mean was calculated. The means of the control group was compared with the test group. There was a significant difference between the means of the two groups. The cortical thickness of the test group was much higher than the control group (Figure 2, 3).

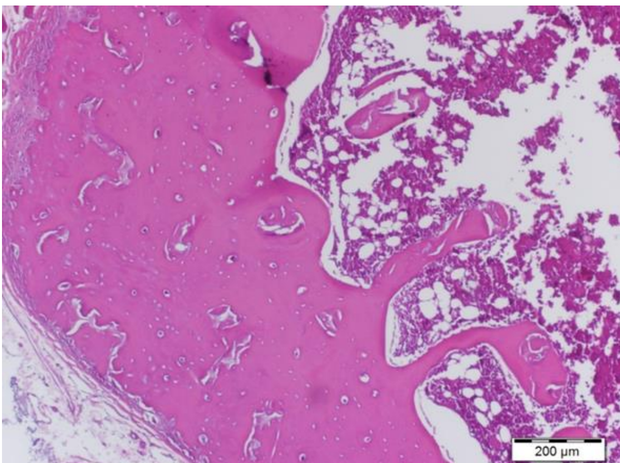


**Figure 2: Tibial cross sections at the level of proximal tibio-fibular joint showing the cortical thickness in the test group - H&E staining (Photomicrograph taken with Olympus BX51 microscope attached with Olympus DP72 digital camera).**

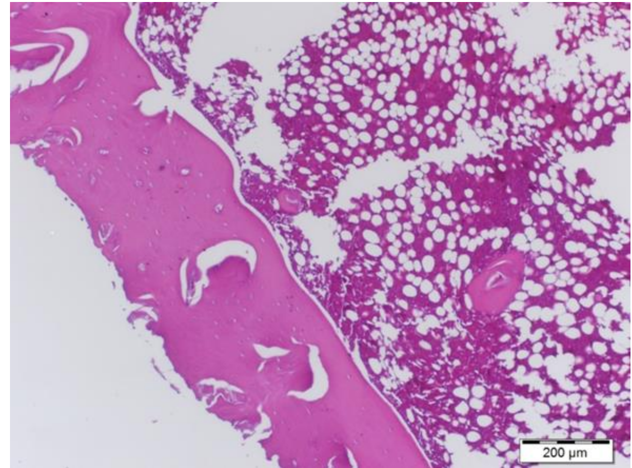


**Figure 3: Tibial cross sections at the level of proximal tibio-fibular joint showing the cortical thickness in the control group - H&E staining (Photomicrograph taken with Olympus BX51 microscope attached with Olympus DP72 digital camera).**

The mean for test group was 528.23 with 95% confidence interval (490.86, 565.60) while the mean for control group was 395.67 with 95% confidence interval (347.60, 443.74) (Table 1). The confidence intervals did not overlap indicating that the two groups differed significantly in the mean (p value <0.0005). The test group had higher mean cortical thickness when compared to the control group (Figure 4).

**Table 1: Comparison of mean cortical thickness in the test group versus control group.**

| Group | Sample size | Mean | 95% confidence interval for the mean | p value |
|---|---|---|---|---|
| Test | 20 | 528.23 | (490.86, 565.60) | <0.0005 |
| Control | 20 | 395.67 | (347.60, 443.74) | |

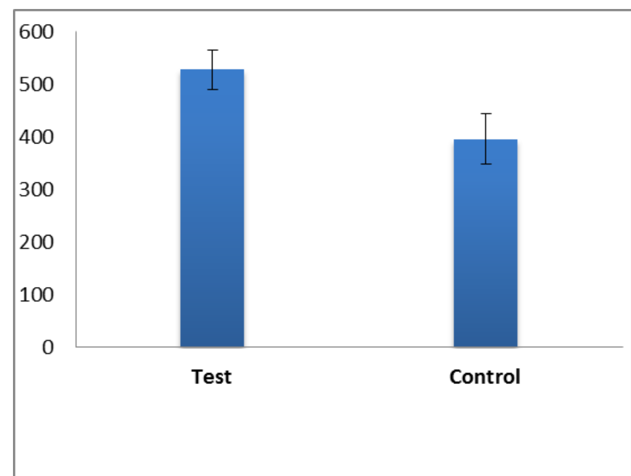


**Figure 4: Comparison of means of cortical thickness in the test group and control group.**

The trabecular bone volume was calculated as percentage of trabecular area relative to the total area of bone marrow cavity. There was not much difference in the trabecular volume between the test group and control group (Figure 5).

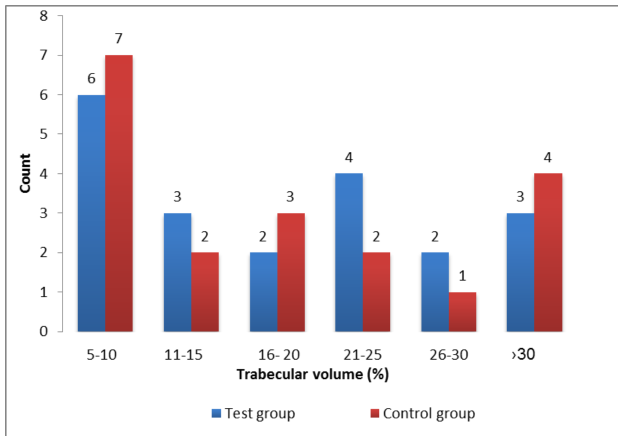


**Figure 5: Comparison of trabecular volume (percentage trabecular area) in the test group and control group.**

## DISCUSSION

Male rats are unsuitable models for osteopenia studies because growth plates do not close in less than 30 months.[17] Ovariectomised rats are accepted animal models of postmenopausal osteoporosis.[18] Ovariectomy of skeletally mature rats leads to a condition similar to menopause. The surgery leads to cancellous as well as endocortical bone loss by increasing the overall rate of bone remodeling and by altering the balance between bone formation and bone resorption. Prior to initiating a clinical trial in a post-menopausal osteoporosis study, it is reasonable to recommence the evaluation of treatment in the 9-month-old ovariectomized female rat.[19] The earliest changes in the cortical bone width and the marrow cavity of the tibial shaft occur 90 to 120 days after ovariectomy.[20] In our study, the ovariectomised rats sacrificed at the end of three months showed significant osteoporotic changes when compared to the sham surgery group.

In animals, hADSCs transplantation by intravenous infusion has shown high safety.[21-24] In our study, none of the animals who were given intravenous infusion died. The animals which were given stem cell infusion showed osteoid formation and presence of osteoblasts indicating new bone formation (Figure 1). The cortical thickness was significantly more in animals which were given stem cell infusion which correlated with the findings reported by earlier researchers,[25] (Figure 2-4). In The trabecular volume did not show a significant increase when compared to the control group (Figure 5) which was in contrast to earlier report.[26]

In the present study, the female Wistar rats were given osteogenesis induced adipose derived stem cells as intravenous infusion 3 months after ovariectomy since it has been reported that osteopenia become progressively pronounced with time upto 100 days.[27] Osteoporosis in the ovariectomised rats was confirmed by comparing with the sham surgery group. It is already established that when osteoporosis sets in, there are decreased numbers of mesenchymal stem cells and they are dysfunctional.[28] Therefore we hypothesized that intravenous injection of osteogenesis induced adipose derived stem cells would lead to osteogenesis and bone repair. hADSCs have become the main type of adult stem cell that is approved for use in humans. hADSC transplanatation has been gradually developed in many countries for the treatment of chronic and degenerative diseases.[29]

The distribution of mesenchymal cells after intravenous infusion is still not fully understood. Previous study in which intravenous infusion of mesenchymal cells derived from the bone marrow was given to 8 month old Fischer F344 rats one week after ovariectomy showed that there was no regeneration of bone tissue.[30] They recommended that it may be necessary to either combine MSCs with an osteoinductive carrier material, or provide an autologous stimulus by expressing a cell differentiation-inducing agent such as BMP or TGFβ into the cells (MSC-based gene therapy) in order to achieve therapeutically significant bone regenerative effects. Accordingly the stem cells were exposed to osteogenic induction medium before intravenous injection.

The rats in the control group and the test group were sacrificed after 35 days to study the effects of intravenous infusion of stem cells. In a previous study done to evaluate the effect of adipose-derived stem cells on bone healing on titanium implant in tibia the rats were sacrificed after 1, 2 and 4 weeks. Their results showed new bone formation in the experimental group.[31] Results of the present study also showed new bone formation in the test group.

The mean cortical bone thickness and trabecular volume are reliable histomorphometric parameters used for the assessment of bone resorption and bone formation.[32] In the present study, the cortical thickness was significantly higher in the test group than in the control group which is indicative of bone formation. But the percentage trabecular area did not show much difference between the test group and control group. The results differed from the previous study done by transplantation of allogenic ADSCs into female Wistar rats with glucocorticoid induced osteoporosis which showed increased percentage trabecular area.[33]Another study done to investigate the efficacy of adult adipose tissue-derived stem cells in restoring bone using an osteoporotic rat model reported that they found a trend for an increase in the bone mineral density and Bone Volume/Trabecular bone Volume (BV/TV).[34]

It is well known that there is a difference in bone growth, modeling, and remodeling in cortical and trabecular bone. Typically, bone growth and modeling improves new bone formation on the bone surface without prior resorption, whereas bone remodeling either conserves or removes the bone in contact with the marrow cavity.[35] Deterioration of the trabecular bone with estrogen loss leads to a reduction in both trabecular bone mass and an irreversible alteration of trabecular bone structure.[36]

Lack of increase in the percentage trabecular area may be due to the differential effect of stem cells on the osteoporotic bone. It can also be dose related as we gave a low dosage of stem cells compared to studies done in immunodeficient mice. Another possible reason for lack of increase in the percentage trabecular araea in the test group is that we sacrificed the animals after 35 days to study the effect. The results may differ if the animals are sacrificed after 3 months of intravenous infusion of hADSCs. More studies will have to be done to evaluate the effect of adipose derived stem cells on osteoporosis.

## CONCLUSION

Intravenous administration of osteogenesis induced human adipose derived stem cells in ovariectomised Wistar rats showed new bone formation and increased cortical thickness. More studies will have to be done to ascertain the potential of stem cells to regenerate the bone in osteoporosis.

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