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Research Article

OVARIECTOMISED AND LONG TERM INDUCED (OLI) RAT MODEL FOR PRECLINICAL ASSESSMENT OF OSTEOPOROTIC THERAPEUTICS

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ABSTRACT

Osteoporosis affects millions of people, especially the aged population and numerous researchers focus on developing novel pharmacotherapeutic agents for the prevention and treatment of osteoporosis. Animal models have become an indispensable part of the preclinical assessment of osteoporotic therapeutics and rat models have been widely preferred. Majority of studies so far reported have used young or short-term induced rat models, but they do not mimic the pathological condition in senile osteoporosis. Hence this study has focused on developing an ovariectomised and long term induced (OLI) aged rat model for the preclinical assessment studies. Long term osteoporosis was induced in Wistar rats by ovariectomy followed by the maintenance period of 10 months. Histological evaluation and Micro-CT analysis have substantiated the extensive bone loss in the OLI rats compared to Control, ovariectomised and short term induced (OSI) rat model. OLI rats also exhibited impairment in the healing of 3 x 1.5 mm femoral cortical bone defect even after 8 weeks of surgery. Histomorphometric analysis indicated that OLI rats exhibited a poor regeneration efficiency (RE) ratio of 0.67 ± 0.2 compared to 0.95 ± 0.1 in the Control group. Chronic bone loss in the OLI rats together with the impaired osteogenic efficacy has been successfully demonstrated which proposes OLI rat models to be utilized for investigating novel pharmacotherapies or novel biomaterials intended for osteoporotic applications.

Keywords: Osteoporosis, Rat osteoporotic model, long-term osteoporosis induction, estrogen deficiency.

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1 INTRODUCTION

Osteoporosis has become a common skeletal disease and usually makes its appearance during the late age¹. At 50 years of age, 4 in 10 women succumb to osteoporosis related fractures and a prior fracture increases further fracture incidence by 10 fold, thus making osteoporotic treatment among the aged

population an orthopaedic challenge². The alleviating number in the elderly population with associated osteoporotic fracture incidence has raised much socio economic concerns too³. New treatment strategies are therefore required to reduce and prevent the onset of disease and thereby incidence of fractures.

An appropriate animal model is indispensable for evaluating the safety and efficacy of any new modality prior to its clinical application. Rat osteoporotic model has been considered the optimal model for osteoporosis research, as it resembles human pathological condition in skeletal responses to depletion of estrogen and pharmacological effects on bone turnover⁴. There are various methods of inducing osteoporosis in rats such as ovariectomy, low calcium diet and immobilization⁵. Aging predisposes the skeleton to excessive bone loss in conditions of senile osteoporosis. Senile osteoporosis is associated with progressive changes in bone remodelling, mainly by decreasing bone formation relative to bone resorption⁶. However literature review suggests that most animal model-based osteoporosis research has been carried out in osteoporotic models induced by ovariectomy in young rats or by short term induction period of 3-6 months. Significant age-related changes were not observed in bone mineral content (BMC) and bone mineral density (BMD) in Sham and ovariectomised rats posts 3-6 months of induction⁷. Kubo *et al.*, has also reported the weak effect of short term induced osteoporosis (30 weeks post ovariectomy) in the mechanical properties and in early phases of bone defect healing⁸. A more appropriate osteoporotic model would be to have long term osteoporosis induced animals, so as to better mimic the chronic bone loss of the aged human clinical pathology. Hence our objective was to develop a

novel model with chronic bone loss induced by ovariectomy followed by a prolonged induction period. It was also of interest to evaluate the effect of long term induction on the bone healing efficacy in the OLI model.

2. MATERIALS AND METHODS:

Ethical statement:

All *in vivo* studies were performed as per the guidelines and recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India and with the approval of the Institute Animal Ethics Committee (IAEC, India).

Animals were housed in individually ventilated cages at $22\pm2^{\circ}\text{C}$ and $55\pm10\%$ relative humidity (RH). Light levels measured (at 1 meter high) less than 300 lx, and a 12:12 hour dark/light pattern was maintained. Animals were fed with standard pelleted rat feed and drinking water *ad libitum*. The health of the animal colony was monitored as per Federation for Laboratory Animal Science Associations (FELASA) guidelines for parasitology⁹, a written protocol developed from international recommendations for bacteriology, protozoology and virology and was stamped negative for infectious agents. The study also followed the Animal Research Reporting of *Experiments* (ARRIVE) guidelines for the execution, evaluation and reporting of the *in vivo* experiments¹⁰.

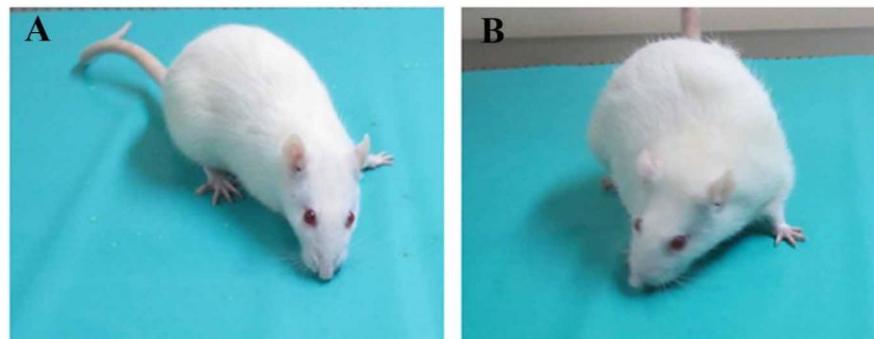


Figure 1: Rat model: (A) and (B) Control rat and ovariectomised and long term induced (OLI) model.

2.1. Bilateral ovariectomy:

Osteoporosis was induced in female Wistar rats - Sctb: WI rats (n=9) (outbred Wistar rats) aged 3 months, weighing approximately 200 g, by bilateral ovariectomy. Animals were further grouped as: ovariectomised and short term induced (four months, n=3) (OSI), and ovariectomised and long term induced (ten months, n=6) (OLI). Animals aged 13 months were included within the Control group (n=6) (figure 1A). Surgeries were performed under general anesthesia using 80 mg/kg ketamine (Anket, Neon Lab, India) and 5 mg/kg xylazine (Xylaxin, Neon Lab, India). Briefly, the surgical site was clipped and prepared with 5% Povidone iodine solution (Betadine, Win Media care, India). The skin and abdominal musculature of the abdominal wall

were incised at the flank level. Using a pair of fine tweezers, the peritoneal fat pad was grasped and exteriorized to view the ovary and uterine horn on each side. The vascular supply to the ovary was clamped using mosquito forceps and the ovary was excised. After clamping the distal region of the uterine horn, a portion of the uterine horn was also excised. The clamps were removed and haemostasis was confirmed. The tissue and fat pad were replaced within the peritoneal cavity using the blunt end of vascular forceps and the skin was closed using 3-0 braided silk (Mersilk, Johnson & Johnson, USA) in a simple interrupted pattern. Povidone iodine solution was applied daily for 7 postoperative days until the sutures were removed. Post operatively animals received subcutaneous injections of

Analgesic-Meloxicam (Melonex, Indian Immunologicals Ltd, India) at 1mg/kg S/C once daily and Buprenorphine (Buprigesic, Neon Lab, India) at 0.05 mg/Kg i/m twice daily for 7 days.

Good nursing support was provided along with the medications, by providing a soft and absorbent bedding to prevent postsurgical infection. Rats were also provided with pre-moistened feed pellets for the first three days after surgery and then high-quality feed (such as sprouted Bengal gram) was provided as soon as the animals recovered, to prevent weight loss and to support their high nutritional demands. Ovariectomised rats were further maintained for 4 months and 10 months to develop OSI and OLI groups respectively (figure 1B).

2.2 Osteoporotic model assessment:

2.2.1. Serum Calcium level analysis:

Serum was isolated from Control, OSI and OLI rats (n = 3 each) at 0, 4 and 10 months following ovariectomy. Serum Calcium concentrations were measured using the kit Arsenazo III method (Cat No: BLT0001 Erba, Germany). Calcium concentration was then compared between the induced and age matched Control groups.

2.2.2. Micro CT evaluation:

Animals (n=3 each from OSI and OLI group) were euthanized at 4 and 10 months respectively, following osteoporosis induction and proximal tibiae were extracted. Muscle tissue was gently scraped off and bones were fixed in 10% neutral buffered formalin. Metaphyseal cancellous bone at the proximal tibiae from Control, OSI and OLI groups were scanned using micro CT desktop scanner CT 40 (Scanco Medical AG, Switzerland) set at 70kVp and 114 μ A. A region of interest (ROI) was chosen by selective contouring of the trabecular bone image slices and three dimensional volumetric model reconstructions were performed using in built software (version - 6.5). Bone parameters such as trabecular number (Tb.N.), trabecular spacing (Tb.Sp.) and bone density were assessed and further evaluated.

2.2.3. Histological evaluation:

For histological analysis formalin fixed bone samples (n=3) were decalcified using 12% Ethylene di-amine tetra acetic acid (EDTA) and underwent a series of dehydration processes in alcohol prior to paraffin embedding. Sections from the paraffin embedded blocks were cut using a rotary microtome (Leica RM 2255, Germany), stained using Hematoxylin and Eosin (H&E) and evaluated by light microscopy (Leica DM 6000, Germany).

2.3. Evaluation of effect of long term osteoporosis induction in the OLI model:

2.3.1. Surgical procedure:

To assess the effect of long term osteoporosis induction on bone healing, femoral bone defects of 3mm x 1.5mm were made in OLI rats (n = 3 rats) and compared with that of the Control animals. Under Xylazine-Ketamine anaesthesia (5mg/Kg and 80mg/kg, respectively) distal femur was exposed through a lateral approach and a bone defect was created with a surgical drill (Marathon, Saeyang microtech, Korea). The muscle flap was then replaced and incision was sutured.

Postoperatively, all animals received antibiotic injections intramuscularly for 7 days. Animals also received subcutaneous injections of Meloxicam (Melonex, Indian Immunologicals Ltd, India) at 1mg/kg S/C once daily and Buprenorphine (Buprigesic, Neon Lab, India) at 0.05 mg/Kg I/M twice daily.

2.3.2. Histological evaluation of bone defect healing:

Animals were euthanized 8 weeks post surgery and the defect sites, along with the adjacent bone, were extracted without disturbing the defect area. Retrieved bone samples were then formalin-fixed and underwent a series of dehydration steps in alcohol, followed by infiltration and embedding in methyl methacrylate (MMA). Plastic embedded blocks were then sectioned using a high-speed precision saw (Isomet TM 2000 Precision Saw, Buehler, USA). Plastic sections were then manually polished down to 70-90 microns using a variable speed grinder/polisher (Ecomet 3000, Buehler, USA) and stained with Stevenal's blue and van Gieson's picrofuchsin¹¹. Histological sections were then micrographed using the light microscope (Leica DM 6000, Germany).

Histomorphometry was performed to quantify bone defect healing in the Control and OLI animals. Consecutive histological sections (n = 3-4 plastic sections) from randomly selected blocks were blindly evaluated using histomorphometry image analyzing software (Leica Qwin, Germany). Regeneration ratio (RE) (ratio of New Bone formed per Total area: [NB/TA]) was calculated from each section to assess the healing efficacy.

2.4. Statistical analysis:

All data included in the study were expressed as mean \pm standard deviation (SD). Results of the biochemical analyses of serum Calcium concentration and estimation of trabecular bone loss based on micro CT scans were evaluated using 2 way ANOVA (with multiple comparisons) in Graph pad prism (version 6.01). Statistical comparisons of histomorphometric data were performed using unpaired t tests. A p-value < 0.05 was considered statistically significant in all analysis.

3. RESULTS:

3.1. Osteoporotic model evaluation:

All animals (Control, OSI and OLI) recovered from surgery without any adverse effect or inflammatory responses.

3.1.1. Evaluation of serum Calcium concentration:

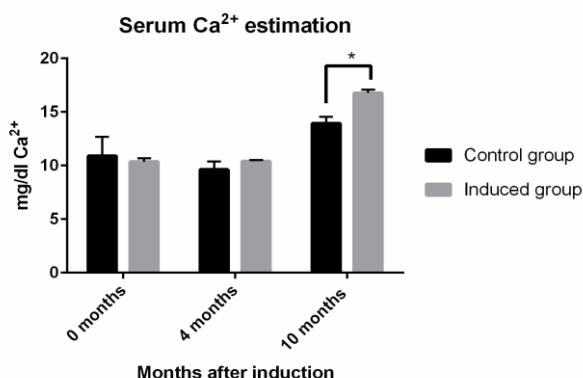


Figure 2: Biochemical analysis: Graphical representation of Calcium concentration in the serum

3.1.2. Evaluation of bone loss using micro CT:

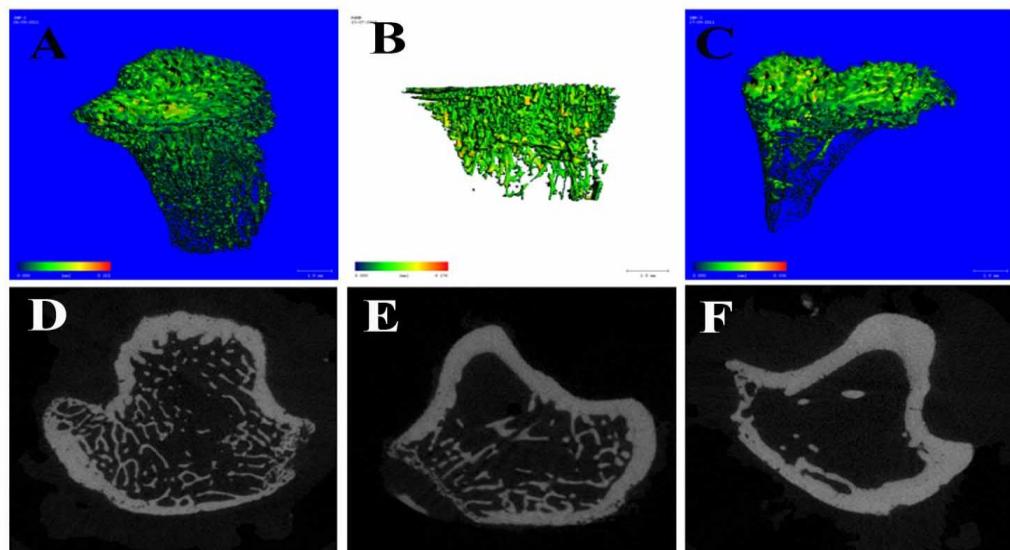


Figure 3: Osteoporotic model evaluation using micro CT scans: (A)-(C) depict the 3D trabecular thickness profile of the proximal tibial metaphysis from Control, OSI and OLI groups. Thinner trabeculae with less trabecular bone volume were evident in OSI and OLI groups. But extensive trabecular disruption was evident in the OLI group. (Color coding represents trabecular thickness - thickness increases from green to red).(D), (E) and (F) depict random 2D slices from Control , OSI and OLI groups indicating the extent of trabecular bone disruption in OLI rats, when compared to Control and OSI rats.

In Control and OSI rats, trabecular bone (represented in greenish yellow color) was seen more prominent and extended downwards from the tibial plateau (figure 3A and 3B respectively). In the OLI rats, trabecular bone was evident only at the proximal tibial plateau, indicative of extensive trabecular disruption and bone loss. Random 2D slices generated from micro CT scans also depicted the extent of trabecular

of the osteoporosis induced rat model post 0, 4 and 10 months of induction compared to age matched Control group. (*- significant, actual p value=0.0218).

Biochemical analyses (figure 2) indicated that serum Calcium concentration in ovariectomised rats was not significantly different from that of the age matched Control group at 0 and 4 months, indicating not much bone loss with short term induction period. Control group rats exhibited a Calcium concentration of 10.91 ± 1.76 mg/dl and 9.632 ± 0.76 mg/dl, whereas the induced group animals exhibited a Calcium concentration of 10.35 ± 0.3 mg/dl and 10.41 ± 0.74 mg/dl at 0 and 4 months respectively. The induced rats exhibited a Calcium concentration of 16.77 ± 3.0 mg/dl post 10 months of induction, which was significantly higher than that of aged matched Control rats, which exhibited a Calcium concentration of 13.94 ± 0.90 mg/dl (p value – 0.0218), indicating extensive bone loss.

disruption among the different groups. In the OSI rat model (figure 3E) trabecular network was evident with slight disruption compared to a mesh-like network in the Control group (figure 3D), whereas in the OLI rat model (figure 3F) a few sprouts of trabecular network was only evident indicative of excessive bone loss compared to other groups.

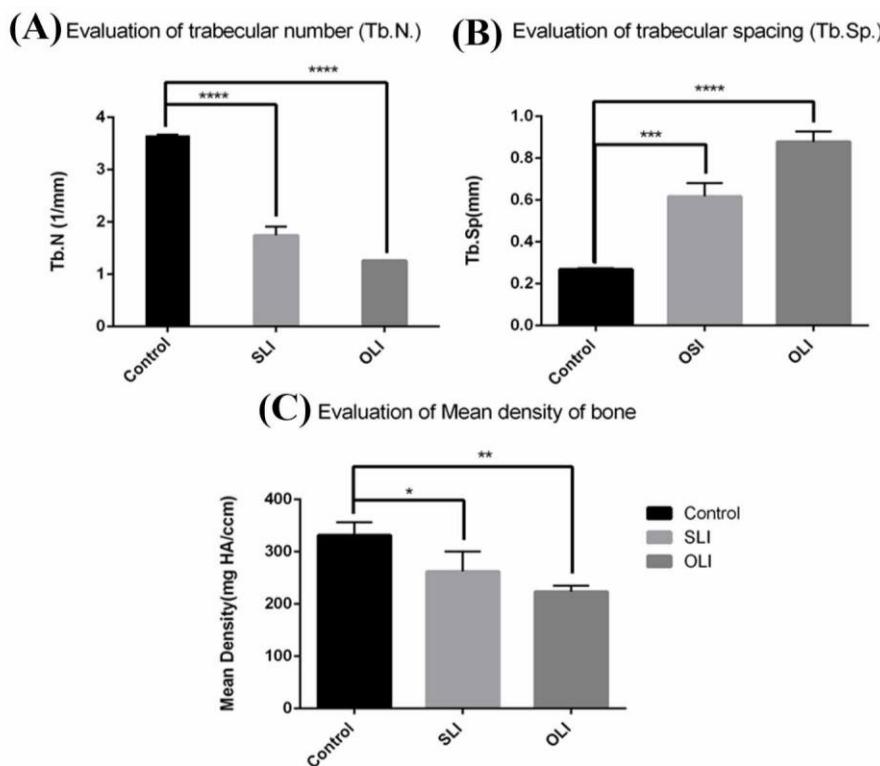


Figure 4: Quantitative evaluation of bone loss using micro CT scans: Decrease in trabecular number (Tb.N.) in the proximal tibial region from Control, OSI and OLI groups are represented in (A). Increase in trabecular spacing (Tb.Sp.) in the proximal tibial region of OLI group, compared to Control and OSI groups is depicted in (B). Figure 4C indicate the decline in bone density (mg HA/cm³) in the OLI and OSI groups, but more significant change was evident in the OLI group, thereby confirming the significance of long term induction period in rats. (Fig A – Control vs. OSI **** p value <0.0001, Control vs. OLI **** p value <0.0001; fig B - Control vs. OSI *** p value 0.0002, Control vs. OLI *** p value <0.0001; fig C - Control vs. OSI * p value 0.0362, Control vs. OLI ** p value <0.0051)

Micro CT evaluation (figure 4A) indicated OSI and OLI rats to have a trabecular number (Tb.N.) of $1.73 \pm 0.17/\text{mm}$ (****p value <.0001) and $1.26 \pm 0.04/\text{mm}$ (****p value <.0001) respectively, compared to Control rats with Tb.N. of $3.65 \pm 0.01/\text{mm}$. Significant decline in Tb.N. was noted in the osteoporosis induced groups. Trabecular Spacing (Tb.Sp.) (figure 4B) was found to be $0.88 \pm 0.03\text{mm}$ (****p value <.0001) for OLI rats and $0.62 \pm 0.02\text{mm}$ (****p value 0.0002) for OSI rats, compared to Control rats with Tb.Sp value of $0.27 \pm 0.03\text{mm}$. Trabecular spacing seemed to increase with advancing induction period and significant high value in the OLI

rats indicate that trabecular bone in the tibial head has been almost replaced with void spaces indicating trabecular disruption. Evaluation of trabecular bone density (figure 4C) also indicated a highly significant bone loss in the OLI animals, compared to other groups. OLI rats exhibited a significantly low mean density of $223.2 \pm 6.5 \text{ mg HA/cm}^3$ (**p value 0.0051) whereas the OSI rats and Control rats exhibited a value of $261.82 \pm 38.21 \text{ mg HA/cm}^3$ (*p value 0.0362) and $331.1 \pm 14.54 \text{ mg HA/cm}^3$. Trabecular bone parameters indicated chronic bone loss in the OLI animals, compared to OSI and Control rats.

3.1.3. Histological evaluation of trabecular bone loss:

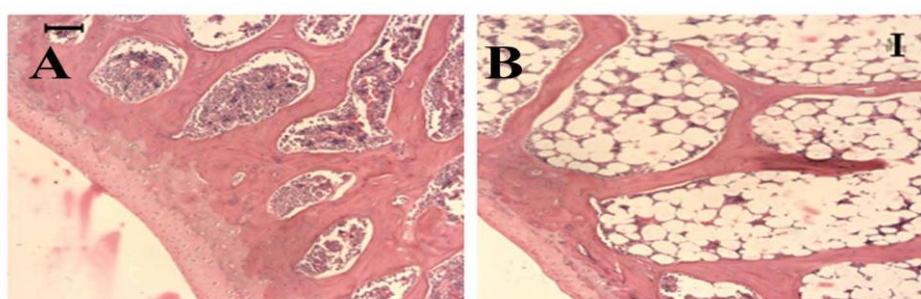


Figure 5: Histological evaluation of bone loss. H&E-stained proximal tibial sections of OSI (A) and OLI rats (B) depict trabecular bone disruption. More void spaces with less trabeculae were evident in the OLI group. (Scale bar equivalent to 100μm).

Histological evaluation of H&E stained sections of the tibial bone depicted a prominent trabecular network in the OSI group animals (figure 5A), whereas, in the OLI animals the trabecular

network was noticeably thinner and surrounded by large void areas, indicating the extent of bone loss (figure 5B).

3.2. Histological evaluation of bone defect healing:

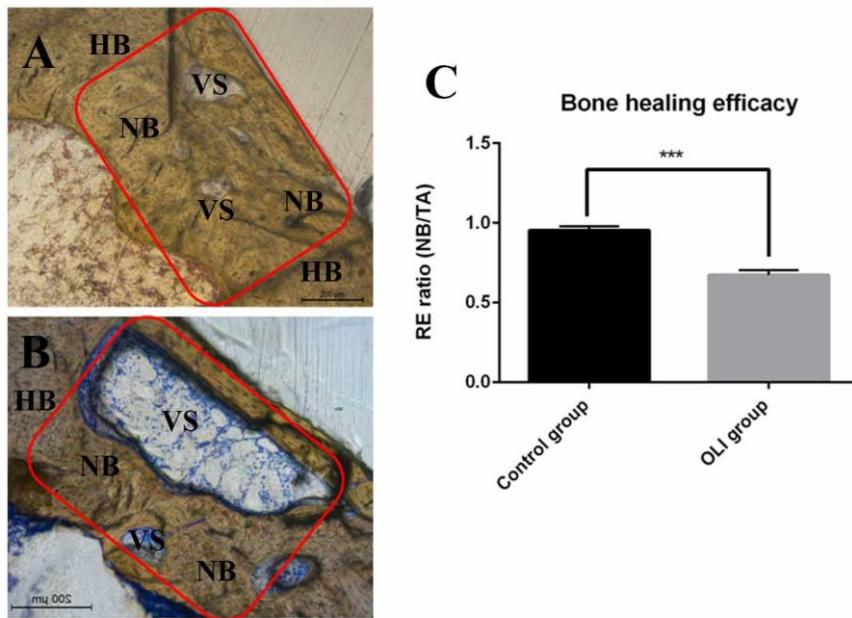


Figure 6: Histological evaluation of bone defect healing: (A) & (B) shows Stevenal's blue and van Gieson's picrofuchsin stained plastic sections depicting *in vivo* healing in Control and OLI rats, 8 weeks after surgery. Large void spaces in the defect area (defect area – framed in red box) in the OLI rats (B) indicates delayed bone healing. In Control rats (A) the defect area was almost bridged by *de novo* osteoid matrix. Histomorphometry analysis (C) in terms of regeneration efficiency ratio indicated impaired healing in OLI animals, compared to Control group. (HB – Host Bone, NB – New Bone, VS – Void Space, TA – Total Area, RE – Regeneration Efficiency = New Bone/Total Area). ((A) & (B) scale bar equivalent to 200 μ m. (C) - *** significant, p value 0.0003)

Histological evaluation carried out at eight weeks indicated reduced bone regeneration efficiency in OLI rats. In Control group (figure 6A), the bone defect area was almost bridged and filled with *de novo* osseous tissue. But in the OLI group, (figure 6B) even after eight weeks of surgery cortical bridging and closure of the defect area was not evident. Loosely arranged osteocytes (immature bone organization) could be distinguished in the *de novo* bone matrix in the control animals. But more void spaces with scarce new bone formation were evident in the OLI rats, indicative of poor healing. Further, histomorphometric analyses (fig 6C) indicated that OLI rats had a significantly lower RE ratio (0.67 ± 0.02) compared to age-matched Control animals ((RE ratio of 0.95 ± 0.0 ; $p = 0.0003$), further supporting the effect of long term induction period in bone healing.

DISCUSSION:

The effect of long-term estrogen deficiency in rat models, to better mimic the clinical situation of senile osteoporosis in postmenopausal women, has not been explored. Most *in vivo* studies employ a short term period (3-4 months) of estrogen deficiency in rats for

osteoporotic induction^{12, 13} and different outcomes have been reported. Aging affects bone quality and has a major influence on skeletal morbidity and mobility¹⁴. Literature suggest that young rat skeletons are not to be considered as an appropriate model for senile osteoporosis, as bone turnover in these animals is primarily modelling (bone formation and bone resorption at specific sites), whereas in human osteoporosis, bone turnover is considered to be remodelling (formation and resorption occur in a cyclic manner at the same site)¹⁵. However, rat skeletal homeostasis is predicted to exhibit a gradual transition from modelling to remodelling with aging suggesting aged rat models to better mimic clinical pathology¹⁶. Therefore research on therapeutic modalities should consider the bone site and age of the rat model to be used. Rats at 6 months old inhibit rapid and linear bone growth as a sign of attaining maturity and maintaining Calcium haemostasis¹⁷. Fukuda and Iida (2004) have predicted 12 months old Wistar rats to exhibit age related bone loss in the proximal tibial metaphysis¹⁸. Accordingly, the significantly increased serum Calcium level in the 10 months osteoporosis induced OLI animals compared to SLI animals suggest the significance of long term induction period in view of

clinical senile pathology. An increase in serum calcium concentration in normal rats at 13 months age has been reported elsewhere, indicating the effect of aging in bone loss^{19, 20}. The OLI models used in the study may be considered to have the etiological factors associated with estrogen deficiency and aging as the experimental animals were 13 months old, post 10 months of osteoporosis induction.

Osteoporosis is reported to mainly affect the areas where bone remodelling is higher²¹, such as the proximal tibial metaphysis. Histological sections of proximal tibiae of OLI rats exhibited a prominent disruption in the trabecular bone, indicating that ovariectomy was successful. Qualitative and quantitative parameters measured using micro CT further substantiated the bone loss. Pathology of osteoporosis in humans is characterised by a reduction in the trabecular number and an increase in the trabecular separation²². Most of the animal models reviewed, exhibited similar trends in trabecular micro-architecture and demonstrated trabecular bone loss²³. Both OSI and OLI rats exhibited a significant bone loss compared to Control animals, but it was well evident that the extent of bone loss increased with an increase of induction period in the OLI rats. Bone density was significantly reduced in the OLI rats supporting the hypothesis of long term osteoporosis induction and bone loss.

Meyer *et al.* reported that prolonged osteoporosis significantly impairs the process of fracture healing²⁴. In ovariectomised rats, alteration in the expression of cytokines and growth factors occurs, which eventually alters the mineral matrix composition of the bone leading to impaired bone healing²⁵. Osteoinductive capacity of bone matrix also decreases with age²⁷. Plastic embedded bone sections stained with Stevenal's blue and van Gieson's picrofuchsin depicted impaired healing ability in the OLI model as the *de novo* bone formed within the defect area exhibited a loose arrangement of the osteoid matrix, resembling islands of woven bone²⁸ with large void space in between and scarce cellular infiltration. Oliver *et al.*, 2013 has also reported the significance of using a long term estrogen-deficient rat model for understanding the pathophysiology of osteoporotic fracture healing in an open fracture model²⁶.

Impaired proliferation and differentiation potential of resident mesenchymal stem cells and induction factors have been reported to delay bone healing²⁹. In short term-induced models, delay in fracture healing is attributed to the accelerated

resorption by osteoclast cells resulting in bone loss, but these models lack the reduction in osteogenic capacity of the bone-forming cells³⁰. Conversely, in our study impaired osteogenic potential was evident in the long term induced osteoporotic model by 8 weeks, whereas the Control rats exhibited a complete bridging of the defect. Conclusive statements regarding the effect of aging on the OLI model can be made by conducting a more extensive study including extended time points and evaluating exclusive age related parameters. More information on the effect of long term induction on the mechanical parameters and molecular pathways may help in better validating the model for preclinical evaluations and further research is warranted.

CONCLUSION

The results of the study show that a long term osteoporosis induction period following ovariectomy in rats, induces chronic bone loss. This is attributed to the combined effect of ovariectomy and aging, thus mimicking the clinical pathology that exists among the geriatric population. The OLI models also exhibited a delayed healing pathology, further validating the osteoporotic model. Such OLI models may be used in future studies to assess the safety and efficacy of drugs intended for the prevention and treatment of osteoporosis. OLI rat models with inherent delayed healing ability may also be suitable for evaluating the *in vivo* osteogenic efficacy of therapeutic implants for addressing osteoporotic skeletal reconstructive surgeries.

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Declaration of Conflicting Interests

The author(s) declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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