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Research Article

STANDARDIZATION OF SPRAGUE-DAWLEY RATS AS A POST-MENOPAUSAL OSTEOPOROTIC MODEL THROUGH BIOCHEMICAL MARKER EVALUATION AND DEXA SCAN

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ABSTRACT: The study aims to evaluate a standardized Sprague-Dawley (SD) rat model for postmenopausal osteoporosis (PMO) research, including the selection of animals, surgical procedures, anesthetic dosage, and supportive care for a speedy recovery, osteoporotic induction, and biochemical and DEXA scan evaluation. Midventral ovariectomy was performed by removing ovaries to simulate a postmenopausal clinical condition in SD rats. The serum markers, estradiol, osteocalcin, and tartrate-resistant acid phosphatase levels in serum were estimated, and PMO status was confirmed with a DEXA scan by evaluating the whole bone mineral density and bone mineral content. The ovariectomized rat (OVX) showed a sharp loss in bone mass and mineral content and serum calcium significantly lowered from 11.21±0.85 mg/dl to 6.34±0.69 mg/dl, magnesium from 2.87±0.43 mg/dl to 2.11±0.31 mg/dl and phosphorus 4.03±0.58 mg/dl to 2.73±0.87 mg/dl respectively. The OVX rats showed a significant reduction in osteocalcin and estradiol values of 1.08±0.68 ng/ml and 67.28±11.46 pg/ml at the end of 6 months, respectively. In OVX animals, the total bone mineral density obtained by DEXA scan was 0.164 ±0.004 g/cm^2 compared to sham control (0.183 ±0.006 g/cm^2). Midventral ovariectomy can be considered an accepted surgical procedure to create an animal model for understanding PMO-associated bone loss. Serum estradiol evaluation along with osteocalcin, calcium, and DEXA scan standardized the status of PMO for detailed study.

Key words: Calcium, Estradiol, Midventral ovariectomy, Osteocalcin, Osteoporosis.

INTRODUCTION

Osteoporosis is a severe metabolic geriatric bone disorder characterized by accelerated loss of bone mass, leading to bone fragility and increased fracture risk (Sozen *et al.* 2017). Older women after menopause are mostly affected and are at the highest fracture risk. Being an old-age disease, it is a significant public health problem worldwide (Salari *et al.* 2021). Osteoporosis is often challenging to diagnose since it lacks any symptoms or health problems. Bone loss occurs when the balance in bone formation and resorption is altered, resulting in a more significant bone loss due to the activity of osteoclasts. Osteoporosis's main features are systemic bone mass reduction and failure of bone homeostasis. There is an imbalance in bone remodeling due to excessive bone resorption relative to bone formation. This imbalance occurs most commonly in postmenopausal women and aged people due to accelerated loss of bone mass (Foger-Samwald *et al.* 2020).

The scientific community is eagerly searching for laboratory animal models to evaluate the biological mechanisms behind the clinical conditions of osteoporosis and many other biological conditions (Barré-Sinoussi *et al.* 2015, Nas *et al.* 2022, Nain *et al.* 2022). Yousefzadeh *et al.* (2020) reviewed that no experimental animal model is ideal for osteoporosis research. The similar mechanism of action in response to estrogen deficiency and associated therapeutic agents between the human and rat skeleton has made the ovariectomized rat a relevant and convenient osteoporosis experimental model, though many related experiments were performed on other animals (González-

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Madariaga *et al.* 2020, Singh *et al.* 2022, Anastasya *et al.* 2022). Most developing countries have limited treatment options, and ordinary people are not concerned about this bone disorder. Hence most osteoporotic patients do not receive timely treatment. This common disorder, a major health issue among the elderly, requires much more attention to find alternate cheap treatment options, including food and nutraceuticals. The ovariectomized rodent model is the most common species used for osteoporosis research (Yousefzadeh *et al.* 2020) due to its affordability (*e.g.*, purchase, breeding, and housing), easy handling, and animal number. The OVX rats exhibit interchangeable characteristics of induced estrogen deficiency or postmenopausal bone loss in humans, especially during the initial phase of osteoporosis (Kharode *et al.* 2008). The age of the experimental animals selected for the study needs to be precisely monitored to evaluate the effects of the surgical procedure, including wound healing and the recovery period.

The present study's main aim is to standardize the Sprague-Dawley (SD) rat model for postmenopausal osteoporosis research through midventral ovariectomy. Because the mid-ventral method of ovariectomy was easy to perform, time-saving, minimally invasive, and single incision site compared to the dorsolateral two-skin incision protocol (Sankar *et al.* 2014). This study emphasizes the essential criteria for selecting the experimental animals, their age group, and number of animals, anesthesia dosage, post-surgical care, antibiotic administration, and hygienic cage support. The cage-side observations, biochemical evaluation, and bone densitometry are commonly studied to confirm the osteoporotic status.

MATERIALS AND METHODS

Andreollo *et al.* (2012) investigated the age relationship between a human and a rat during the social maturity phase. Based on the age relationship, female SD rats of 6-12 months of growth were selected for the study. Twenty-four female SD rats (weighing approximately 280±30 g) were procured from the animal house facility, Department of Biochemistry, University of Kerala. Pre-operative management includes the selection of healthy animals for the experiment. They were housed in standard environmental conditions with ambient temperature (25±1°C), relative humidity (55±5%), and a 12/12 h light-dark cycle. The animals were provided with a standard rat pellet diet and ad libitum water. All the experiments were carried out per the CPCSEA guidelines with the institute's animal ethical committee approval for conducting animal experiments (IAEC-2-KU-01/2018-19-BCH-AAR).

Animal preparation

Experimental animals were weighed and properly restrained. The ketamine-xylazine combination was used for anesthesia, and the dosage was standardized as 90 mg/kg and 10 mg/kg (Van Pelt 1977) respectively, and anesthetic drugs were administered intraperitoneally. The majority of the animals, nearly 80%, were anesthetized within 10-15 minutes of injection. The level of anesthesia was checked and confirmed with interdigital and tail-tip pinch responses (Yurie *et al.* 2017). The surgical site was shaved and sterilized with alternating washes of 10% betadine and 70% surgical spirit. Sterile conditions were maintained throughout the surgical procedure, and sterile drapes were used to place the surgical instruments and anesthetized animals. The animal was placed in a sterile surgical bag with drapes for the surgical procedure. Thermal support was provided to the animals throughout the surgery and continued until they had a complete recovery.

Midventral ovariectomy

Ovariectomy was started by anesthetizing the rats using a combination of xylazine and ketamine, as described above. The midventral area was caudal to the third pair of mammary papillae was shaved and cleaned with a disinfecting solution. Using the scalpel blade, a skin incision was made carefully, and then a muscle incision was performed to access the peritoneal cavity. The adipose tissue was carefully pulled out using a blunt retractor and the ovaries embedded in the fat tissue were bowed out from the incision site. The ovaries were exposed, and a suture knot was placed at the distal uterine horns on both sides of the ovaries to prevent bleeding. Gently the ovaries were cut and removed, as shown in (Fig. 1). The peritoneum and the white line were closed with a running absorbable 3.0 absorbable sutures of vicryl (cutting edge). The skin was sutured with an interrupted absorbable 4.0 thread (Khajuria *et al.* 2012, Lipton 2004). After the completion of skin suturing, betadine ointment was applied to prevent infection. The rats were covered with gauze dressing to avoid hypothermia (www.fda.gov 2015). After the procedure, the antibiotic drug ceftriaxone @ 50 mg/kg and the anti-inflammatory drug meloxicam @ 1 mg/kg were administered to assist in wound healing, relieve inflammation and pain, and prevent muscle cramps (Bekker *et al.* 2018).

The animals were wrapped in a sterile cloth and placed in a clean cage, and antibiotic injections were repeated for 3 days. Sterile conditions and an aseptic environment are mandatory throughout the procedure to prevent infections and the animal's total health (Sophocleous and

Idris 2014). In the sham control rat (group 1), an incision was made, and the ovaries were pointed out and then placed back in the lower abdominal cavity and sutured properly. The 24 SD rats were grouped into three; 6 in sham control rats (group I) 12 OVX rats (group II) and 6 normal control rats (group III).

Post-operative monitoring

After analgesic administration, the animals were left undisturbed in a clean cage and continued to provide the warmth of 27-30^0 C until they were fully conscious. Animals were monitored daily for the next 3-5 days for any signs of pain or discomfort. Povidone iodine ointment was applied twice daily to minimize possible infections and to enhance wound healing (Bigliardi *et al.* 2017). The ovariectomized rats were housed individually in polypropylene boxes with bedding made of sterilized cotton fabric for 10-14 days.

Physical examination

The physical examination is mandatory for understanding the recovery of animals after ovariectomy, which includes assessment of the overall appearance, posture, and mobility pattern, of the ovariectomized rats during the study.

Serum mineral and biomarker study

Blood was collected from the saphenous vein or jugular vein (Parasuraman *et al.* 2010), and serum was separated by centrifuging the clotted blood sample at 3000 rpm for 15 mins (Woo *et al.* 2013). Serum calcium, phosphorus, and magnesium were estimated as per kit manufacturers' instructions (Agape Diagnostics Ltd, Kerala, India). A rat estradiol ELISA kit was obtained from KINESISDx (K11-0175), Rat osteocalcin (KLR0270), and Rat Tartrate-resistant acid phosphatase 5b (TRACP-5b) was quantified as per kit manufacturers' instructions (Krishgen biosystems. Mumbai, India).

Bone DEXA scanning

The dual-energy X-ray absorptiometry (DEXA) scan was used to take images of the body using a minimal dose of ionizing radiation (Messina *et al.* 2020). The experimental rats were anesthetized before the scanning procedure and kept in a midline alignment position in the scanner bed. All the animals were routinely scanned on a GE Healthcare Lunar Prodigy. For the Prodigy, Encore software (GE Healthcare, Madison, WI), version 9.2 was used to acquire scans, and version 11.4 was used for detailed analysis (Morrison *et al.* 2016).

Statistical analysis

Graph Pad Prism 5 software (Graph Pad Software Inc.) was used to calculate the standard deviation, and two-way analysis of variance (ANOVA). The Pearson correlation coefficient and p values < 0.05 were regarded as significant (Bewick *et al.* 2003). Values expressed are means of six replicate determinations standard deviation.

RESULTS AND DISCUSSION

Midventral ovariectomy is one of the surgical procedures performed to induce postmenopausal osteoporosis. The midventral incision was technically easier, minimally invasive, less time-consuming, and easy to locate the uterine horn and ovary which is embedded in fat. There is no significant impairment is reported including in OVX rats, such as deficit in gait, overall mood, and spatial ability (Sankar *et al.* 2014). Since the pathophysiology and etiology of osteoporosis are very complex and multifaceted, standardizing an ideal experimental rodent model is vital for dynamic osteoporosis research. The ovariectomized rat is well characterized by the human mammalian system and shares estrogen deficiency-induced (postmenopausal) bone loss in women (Khajuria *et al.* 2012). The estrogen deficiency generates an imbalance in bone formation and resorption mechanisms. The number of osteoclast increases compared to osteoblast cells, enhancing bone turnover and resulting in rapid bone mineral density loss.

After midventral ovariectomy, the overall cage-side observations and physical examination of OVX are closely monitored (Table 2). As post-operative care, the administration of analgesics was provided which is mandatory and does not cause any physiological alteration to the experimental animals (Pogatzki-Zahn *et al.* 2017). The overall weights of OVX rats were monitored regularly as a significant weight loss was monitored after surgery. After OVX, from the period of 45 days, the animals gradually started to gain weight and become fatty as time progresses to 6 months; at the same time, a few exceptions were also noted (Table 3).

The assessment of overall appearance, posture, and mobility pattern, which are an inevitable part of physical examination. However, during osteoporosis, these movements become very feeble. The accumulation of debris and waste in the hair coat results in a waxy yellow coating around the hair follicles, particularly in the back and upper neck regions were observed during the study. Dirty hair can be considered one of the early signs of aging-related complications as older rats cannot groom their upper back regions (Phillips *et al.* 2010). Usually, the hair coat is flat with a glossy sheen, but in osteoporosis,

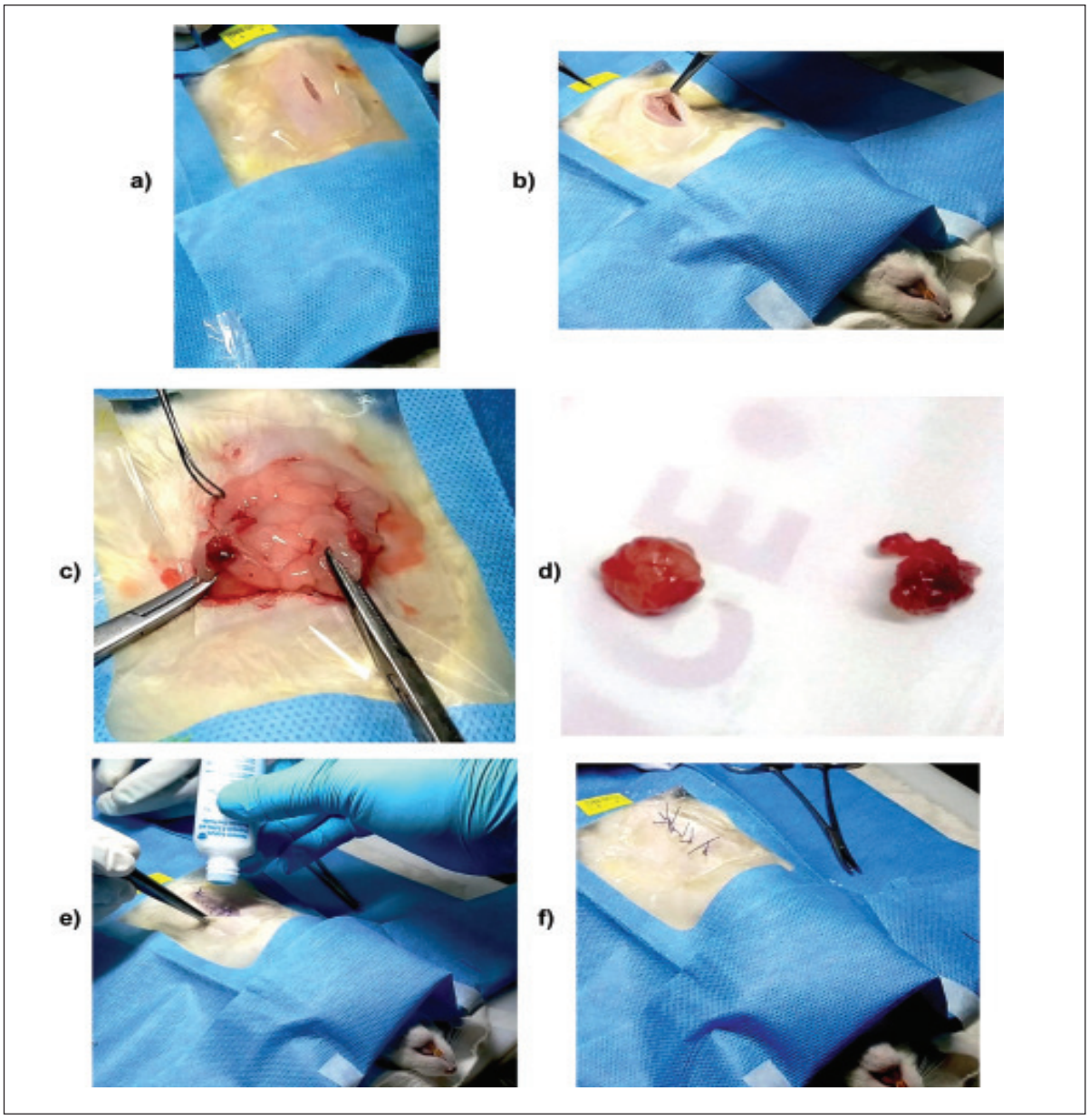

Fig. 1. Mid-ventral ovariectomy. [a) skin incision b) muscle incision c) Identification of both the ovaries d) excised ovaries e) muscle suturing and wound seal application; f) skin suturing].

the animal is more irritated, and hair falls, and dermatitis is also clearly visible (Shaheen *et al.* 2019) (Fig. 2). Small fissures and wounds are also observed in the interdigital space of the legs. Facial features such as sharp and triangular appearances change to blunt and irregular shapes visible in the study.

A healthy tail is always free of fecal staining and discoloration, but in OVX animals, the tails are permanently colored with fecal particles and debris was observed during the study. Abnormal vocalizations or sounds are common symptoms of pain and distress, but they were not noticed during the experimental period. The healthy rat stands or walks similarly to humans (Funato *et al.* 2017). However, in osteoporosis, their posture deteriorates, resulting in arching of the backbone, similar to a hunched position, which drags the abdomen into proximity. Being highly energetic and alert animals, they will constantly roam the cages. With the advance of age, active locomotion becomes a tedious job, resulting in crawling and always being in a resting condition. The muscular tonicity, another notable feature, helps the rats make swift and brisk movements. During osteoporosis,

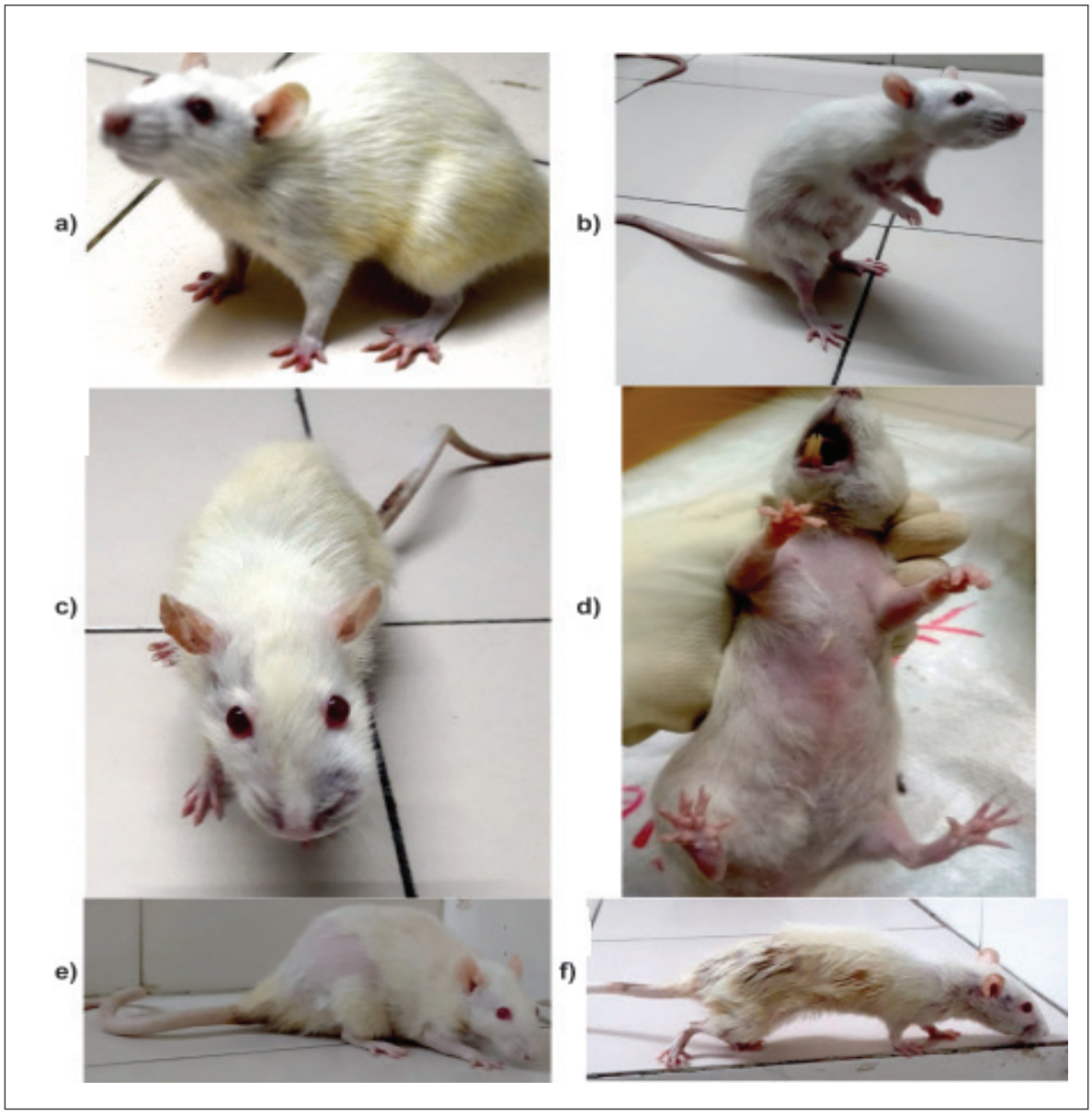

Fig. 2. Physical changes observed in ovariectomized rats. [a) Small fissures and wounds in the interdigital space of the legs b) arching of the backbone c) blunt and irregular facial bone appearance d) severe hair shedding e) faecal staining and discoloration at urogenitial region f) loss of muscle mass at abdominal, chest and leg regions].

the muscle mass in the legs and arms disappear completely. Simple hand palpation procedures can feel skin and no muscle in OVX animals (Phillips *et al.* 2010). Due to the loss of abdominal muscle mass, ribcages are easily visible during severe osteoporotic conditions and are observed in the present study. Some neurological symptoms, such as loss of concentration while grooming and lack of confidence in self-defense, and osteoporosis associated obesity (Ezzat-Zadeh *et al.* 2017) are also observed during the study.

Serum calcium and magnesium were monitored along with phosphorus to confirm the OVX conditions, and the values showed a sharp decrease and are summarised (Table 4). The bone mineral quantifications are very relevant for osteoporosis evaluation because calcium phosphate forms the main constituent of the hydroxyapatite framework. This mineral matrix, along with extracellular protein, forms the structural backbone of bone, the bone mineral that strengthens the bone cells to withstand mechanical resistance (Bonjour 2011). The serum calcium

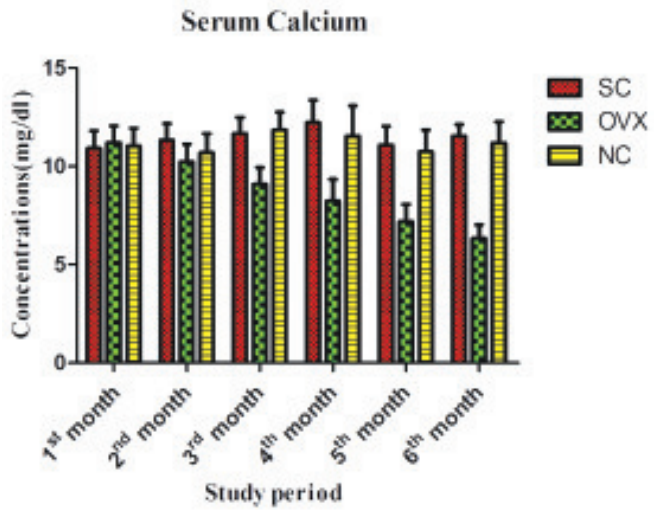

Fig. 3. Serum calcium evaluation after ovariectomy. [Values expressed as mean ± SEM (n=6, and $p < 0.05$). OVX: Ovariectomized rat, NC: Normal control, SC: Sham control, mg/dl- milligrams per deciliter].

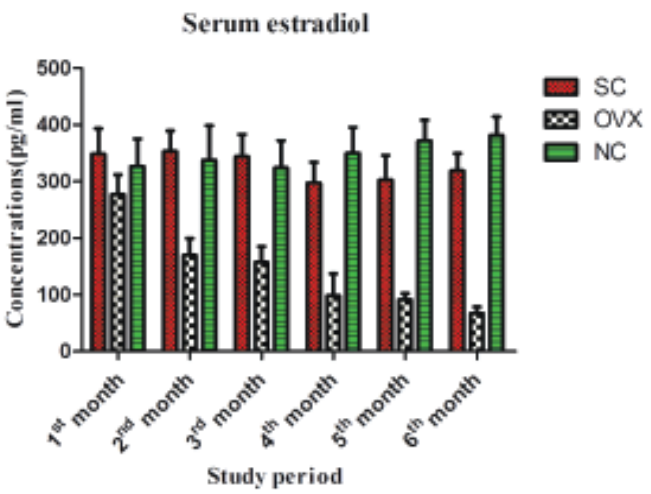

Fig. 4. Serum estradiol evaluation after ovariectomy. [Values expressed as mean ± SEM (n=6, and $p<0.05$). OVX: Ovariectomized rat, NC: Normal control, SC: Sham control, mg/dl- milligrams per deciliter].

Table 1. Cage side observations and physical examination of experimental rats after ovariectomy.

Sl No.	Parameters	Observations	
		OVX rats	Sham control
1	Fur appearance	Irritated, hair fall	Normal
2	Abdominal distension	Not normal	Normal
3	Eyes and ears consistency	Healthy	Healthy
4	Pupil diameter	Not normal	Normal
5	Colour of urine	Yellowish	Pale yellow white
6	Consistency of the faces	Greyish black	Black
7	Condition of teeth	Dental malocclusion	Normal
8	Breathing abnormalities	Normal	Normal
9	Gait	Weak	Healthy
10	Vaginal orifice	Yellowish stains present	Normal
11	Temperature	Normal	Normal
12	Food and water intake	Less than normal	Normal
13	General physique	Seems to be lazy	Good
14	Coma	Not present	Not present
15	Drowsiness	Present	Not present
16	Tremor	Present	Not present
17	Sedation	Present	Not present
18	Facial features	Irregular	Normal
19	Muscle weakness	Present	Absent
20	Death	Alive	Alive

Table 2. Body weight monitoring during experimental period.

Experimental period	SC (g)	OVX (g)	NC (g)
1st month	267.33±22.51	255.83±20.42	278.33±17.51
2nd month	279.17±16.08	269.5±22.21	288.67±25.86
3rd month	289.33±20.82	278.17±19.96	295.5±21.03
4th month	292.83±23.16	291.83±16.42	303.17±20.51
5th month	302.67±21.62	308.5±19.04	307.83±18.83
6th month	312.17±19.61	321.33±22.20	311.5±23.16

[Values expressed as mean ± SEM (n=6, and $p < 0.05$). OVX: Ovariectomized rat, NC: Normal control, SC: Sham control, g: grams].

Table 3. Serum mineral evaluation of experimental rats after ovariectomy.

Experimental period	Magnesium (mg/dl)		Phosphorus (mg/dl)			
	SC	OVX	SC	OVX	SC	OVX
1st month	3.00±0.47	3.05±0.32	3.25±0.40	5.94±0.56	5.20±0.63	6.28±0.44
2nd month	2.94±0.33	2.92±0.47	2.79±0.59	6.07±0.33	4.89±0.60	6.40±0.58
3rd month	3.04±0.34	2.64±0.39	2.94±0.33	6.13±0.68	3.80±1.31	6.35±0.35
4th month	2.89±0.27	2.42±0.33	3.05±0.39	6.18±0.31	3.46±1.26	6.32±0.24
5th month	2.76±.32	2.26±0.30	2.89±0.26	6.12±0.36	3.05±0.60	6.12±0.55
6th month	2.95±0.24	2.11±0.31	2.86±0.39	6.22±0.47	2.73±0.87	6.01±0.57

[Values expressed as mean ± SEM (n=6, and $p < 0.05$). OVX: Ovariectomized rat, NC: Normal control, SC: Sham control, mg/dl-milligrams per deciliter].

Table 4. Serum marker evaluation after ovariectomy.

Experimental period	TRACP-5b (mU/ml)			OCN (ng/ml)		
	SC	OVX	NC	SC	OVX	NC
1st month	15.03±3.27	20.38±3.20	17.88±2.25	17.15±3.24	18.44±3.41	19.73±4.0
2nd month	19.54±2.14	13.53±3.45	15.20±2.15	16.82±2.73	14.53±4.38	19.06±2.81
3rd month	16.37±1.87	9.41±1.98	16.54±1.64	17.65±2.28	11.73±2.21	18.56±2.18
4th month	19.38±2.38	7.10±1.02	14.58±1.48	18.32±2.26	8.95±2.94	15.56±3.14
5th month	17.26±1.65	6.36±0.87	16.40±3.61	15.23±2.90	3.10±0.97	16.23±3.03
6th month	17.88±2.91	2.99±1.26	18.02±2.85	14.65±2.67	1.08±0.68	15.29±4.19

[Values expressed as mean ± SEM (n=6, and $p < 0.05$). OVX: Ovariectomized rat, NC: Normal control, SC: Sham control, mg/dl- milligrams per deciliter].

levels were found to be 11.21±0.85 mg/dl in OVX rats in the first month, further significantly reduced to 9.50±1.19 mg/dl, 8.15±0.94 mg/dl, 7.40±1.11 mg/dl, 7.19±0.88 mg/dl and 6.34±0.69 mg/dl for the 2nd, 3rd, 4th, 5th, and 6th months, respectively (Fig. 3). During osteoporosis, bone remodeling, and bone resorption can induce the release of calcium from the bone cells into the total serum calcium pool of the body (Si *et al.* 2020). Serum magnesium followed the same pattern as serum calcium, which was also found to have a similar mechanism to calcium. It was found to be normal range during the initial period of ovariectomy (3.05±0.32 mg/dl), after which magnesium was remarkably lost to 2.11±0.31 mg/dl. However, the phosphorus exhibited an inverse pattern, i.e., in the range

Table 5. Total BMD and BMC evaluation after ovariectomy in SD rats.

Regions	Sham control BMD (g/cm^2)	Osteoporotic BMD (g/cm^2)	Sham control BMC (g)	Osteoporotic control BMC (g)
Head	0.302±0.013	0.290±0.010	-	-
Arms	0.147±0.023	0.114±0.029	0.63±0.33	0.33±0.17
Legs	0.153±0.004	0.134± 0.003	1.27±0.50	2.1±0.66
Trunk	0.160±0.006	0.132± 0.007	3.63±1.05	1.93±0.38
Ribs	0.151±0.008	0.116±0.007	-	-
Spine	0.165±0.008	0.140±0.006	-	-
Pelvis	0.161±0.005	0.134±0.011	-	-
Total	0.183±0.006	0.164±0.004	7.93±0.32	6.97±0.21
TBLH	-	-	5.53±0.23	4.4±0.53

[Values expressed as mean ± SEM (n=6, and $p < 0.05$). BMC: bone mineral content, BMD: bone mineral density,TBLH: total body less head, $g/cm^{2:}$ grams per square centimeter, g: grams].

of 5.20±0.63 mg/dl to 2.73±0.87 mg/dl (Table 4). Serum mineral composition showed a significant reduction after ovariectomy.

One of the notable variations observed during the study was an increase in tail skin temperature compared to control animals during the initial stage of ovariectomy. Menopausal hot flushes are commonly reported in humans; Kobayashi *et al.* (2000) reported the same in female SD rats. In the menopause stage, in addition to the above changes, a decline in estradiol and progesterone was also reported, which accelerates the decline in osteoblast activity, reduces bone matrix formation, and increases bone resorption due to the cessation of the regular estrus cycles (Seifert-Klauss *et al.* 2010). Serum estradiol level exhibited a significant decrease in OVX animals from the first month (277.01±34.75 pg/ml) onwards and reached 67.28±11.46 pg/ml on the 6th month of evaluation (Fig. 4).

TRACP 5b has been used as a potential marker of bone resorption; the osteoclast enzyme is frequently found at osteocyte lacunae and resorption pits to promote bone turnover and remodeling (Solberg *et al.* 2014). TRACP-5b showed an increased value during the initial period of ovariectomy (20.38±3.20 mU/ml) and then reduced to (2.99±1.26 mU/ml) levels on the 6^{th} month of evaluation. Osteocalcin is seen in the bone matrix and released into the circulation during bone resorption; hence it can be considered a specific bone turnover marker. Osteocalcin values were very high in ovariectomized rats, especially from the 4^{th} to 6^{th} month, with a significant decrease ranging from 8.95±2.94 ng/ml to 1.08±0.68 ng/ml where observed (Table 4). According to Kuo and Chen (2017), as a bone remodeling biomarker, serum OCN can be used to assess the progression of osteoporosis and predict the fracture risk in elderly persons, particularly in postmenopausal women. The entire serum marker showed a total decrease in OVX animals at the end of the sixth month study.

After OVX, the animals significantly reduced bone mineral density (BMD) and bone mineral content (BMC). The arms, spine, and pelvic regions were the most severely affected skeletal sites in OVX rats, which are more vulnerable to fractures (Cummings and Melton 2002). The DEXA scan report revealed that the arms, spine, and pelvic regions were the most severely affected skeletal sites in OVX rats, with lower BMD and BMC than sham control animals. Compared to the sham control (0.183±0.006 g/cm^2), the osteoporotic animal showed a total BMD of (0.164±0.004 $g/cm^{2)}$, and the detailed report of each region is summarised (Table 5). The BMC also exhibited sharp linearity with BMD values. However, the BMC value of the leg region is quite exciting and was found to be higher in osteoporotic animals (2.1±0.66 g) than in control animals (1.27±0.50 g). The total body-less head (TBLH) was also monitored and found to be 5.53±0.23 g for control and 4.4±0.53 g for osteoporotic animals.

CONCLUSION

Ovariectomy is an effective method to induce osteoporosis in laboratory animals. However, the success of the ovariectomy depends on post-operative care, including proper handling, keeping the wound clean and dry and antibiotic support. The estimated serum calcium,

magnesium, and phosphorus significantly decreased after ovariectomy and supported the DEXA scan results. Furthermore, the serum estradiol exhibited a significant reduction in OVX animals, confirming the remarkable effect of ovariectomy. So, the present study inveterate that midventral ovariectomy could successfully induce osteoporosis in SD rats within 6 months without administering other drugs or dietary restrictions. The bone turnover marker (OCN), resorption marker (TRACP-5b), and DEXA scan were used to confirm the osteoporotic status of the OVX animals. Hence, the DEXA scan can be appraised as the most accepted diagnostic procedure to evaluate postmenopausal osteoporosis status in clinical research.

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