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ORIGINAL ARTICLE

An animal model in rat for secondary osteoporosis: the effect of gonadotropin-releasing hormone (GnRH) agonists, low calcium diet, and immobilization on tartrate-resistant acid phosphatase 5b (TRACP-5b) and procollagen type 1 N-terminal propeptide (PINP) levels

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ABSTRACT

Aim Osteoporosis is a global health concern characterized by reduced bone density, microarchitectural changes, and an increased risk of fractures. The prevalence of osteoporosis is rising, particularly in developing countries. In Indonesia, the incidence of upper femur fractures due to osteoporosis is notably high. As human studies are limited, animal models are crucial for investigating osteoporosis, with several methods used to induce the condition. This study proposes an alternative model using leuprolide acetate, gonadotropin-releasing hormone (GnRH) agonist, combined with a low-calcium diet and immobilization, to induce secondary osteoporosis in animals.

Methods This experimental study employed a post-test-only control group design with 12 groups of Wistar rats (*Rattus norvegicus*). Three control groups received no treatment, while nine experimental groups were administered leuprolide acetate along with a low-calcium diet and immobilization. The study measured tartrate-resistant acid phosphatase 5b (TRACP-5b) and procollagen type 1 N-terminal propeptide (PINP) levels in rat bone tissue.

Results Significant increase in TRACP-5b and decrease in PINP levels were observed on days 21, 28, and 35 in the treated groups. Post hoc analysis revealed significant differences between the treatment groups.

Conclusion The experimental model successfully demonstrated a reliable and reproducible method for inducing secondary osteoporosis. Elevated TRACP-5b and reduced PINP levels indicate increased osteoclast activity and decreased osteoblast activity resulting from excessive bone remodelling. The combination of leuprolide acetate, a low-calcium diet, and immobilization is an effective and alternative method for inducing secondary osteoporosis in experimental animals.

Keywords: calcium, immobilization, leuprolide, osteoporosis

INTRODUCTION

Osteoporosis is a significant global health issue characterized by decreased bone density and microarchitectural deterioration, leading to increased bone fragility, reduced bone strength, and a heightened risk of fractures (1,2). The prevalence of osteoporosis is rising, particularly with the growing elderly population, and is increasingly recognized as a significant public health concern, especially in developing countries (3). In Indonesia, for example, the Ministry of Health reported in 2010 that the incidence of upper femur fractures due to osteoporosis was 200 cases per 200,000 people by the age of 40 years (4).

Advances in genetic research have enabled the creation of animal models with specific hereditary traits by manipulating or disabling certain genes (5). Genetically modified mice, for instance, can replicate early onset osteoporosis, thus shortening experimental durations. However, the high cost, complexity, and technical requirements of such models limit their widespread use (6).

Another approach to inducing osteoporosis in animals is to simulate androgen deficiency, which has been shown to affect bone microarchitecture and density. In particular, treatments involving gonadotropin-releasing hormone (GnRH) agonists, such as leuprolide acetate, can cause osteopenia in mice (7). Immobilization is another method to induce bone loss in an-

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imals, where bone mass decreases due to a halt in periosteal bone formation and increased endosteal resorption (8).

Given the complex nature of osteoporosis, a combined treatment approach is often more effective than single methods in mimicking the condition's mechanisms.

The aim of this study was to explore an alternative model combining leuprolide acetate, a GnRH agonist, a low-calcium diet, and immobilization to induce secondary osteoporosis in rats. While each of these treatments has been shown to influence bone density independently, the combined effect is hypothesized to produce a more pronounced osteoporotic phenotype.

MATERIALS AND METHOD

Materials and study design

This study was conducted from January to March 2025 at the Animal Research Laboratory, Faculty of Medicine, and the Physiology Laboratory, Faculty of Medicine, Universitas Brawijaya.

This study employed an experimental design with a post-test-only control group approach. The primary objective was to evaluate the effects of administering GnRH agonists (leuprolide acetate) combined with a low-calcium diet and immobilization on the development of a secondary osteoporosis animal model in rats. The key parameters measured included tartrate-resistant acid phosphatase 5b (TRACP-5b) and procollagen Type 1 N-terminal propeptide (PINP) levels in bone tissue. The sample size was calculated using the Federer formula (9) to estimate the required number of subjects based on power analysis. The formula used is as follows:

$(n - 1)(t - 1) \geq 15$ where: n = required sample size per group, t = number of treatment groups

$$(n-1)(12-1)\geq 15$$
$$(n-1)(11)\geq 15$$
$$11n-11\geq 15$$
$$11n\geq 26$$
$$n\geq 26/11$$
$$n\geq 2.36$$

In this study, there were 12 treatment groups (Table 1). Solving the equation yields a required sample size of 3 rats per group. To avoid a lack of sample size, each group consisted of 4 rats. Therefore, a total of 48 rats were included in this study. The rats were randomly assigned to either the control or experimental groups. The inclusion criteria specified male Wistar rats, aged 12 weeks and weighing approximately 250 grams, which were in good health and showed no physical impairments. The exclusion criteria included male Wistar rats with extremity disabilities, infections, or those that died during the study.

All protocols were approved by the Health Research Ethics Commission of General Hospital Saiful Anwar, with ethical approval number 400/358/K.3/102.7/2024, which was issued on 9 December 2024.

Methods

The animal subjects that met the inclusion criteria were acclimatized for a period of 7 days in a controlled environment with a temperature of 22-24 °C, a 12-hour light/dark cycle, and access to standard laboratory food and water ad libitum.

Table 1. Study treatment groups and protocols

Group	Treatment	Duration (days)
Group 1 (Control)	Rats without treatment, fed a standard diet	21
Group 2 (Control)	Rats without treatment, fed a standard diet	28
Group 3 (Control)	Rats without treatment, fed a standard diet	35
Group 4	Rats injected with Leuprolide acetate	21
Group 5	Rats injected with Leuprolide acetate	28
Group 6	Rats injected with Leuprolide acetate	35
Group 7	Rats received Leuprolide acetate combined with a low-calcium diet	21
Group 8	Rats received Leuprolide acetate combined with a low-calcium diet	28
Group 9	Rats received Leuprolide acetate combined with a low-calcium diet	35
Group 10	Rats receiving Leuprolide acetate, a low-calcium diet, and limb immobilization	21
Group 11	Rats receiving Leuprolide acetate, a low-calcium diet, and limb immobilization	28
Group 12	Rats receiving Leuprolide acetate, a low-calcium diet, and limb immobilization	35

During the acclimatization period, the animals were monitored for health and behaviour to ensure they had adapted appropriately to the environment before commencing the experimental procedures. After acclimatization, the animals were assessed for overall health and subsequently assigned to their respective treatment groups as predefined in the study protocol.

GnRH agonist administration. Leuprolide acetate (KalbeMed, Indonesia) was administered subcutaneously at a dose of 75 mg/kg body weight. The administration schedule varied according to the treatment group. The fourth group received Leuprolide acetate for 21 days, the fifth group for 28 days, and the sixth group for 35 days. For the subsequent groups, Leuprolide acetate was combined with a low-calcium diet for 21, 28, or 35 days, in groups 7, 8, and 9, respectively. Lastly, in groups ten, eleven, and twelve, the same treatment of Leuprolide acetate, low-calcium diet, and limb immobilization was applied for 21, 28, or 35 days.

Low-calcium diet. A low-calcium diet containing <1% calcium to induce calcium deficiency, a known factor in the development of osteoporosis, was given to the treatment group, which was set to receive a low-calcium diet.

Limb immobilization. Limb immobilization was performed immediately after recovery from anaesthesia using plaster of Paris (POP). The left hind limb was flexed at the knee joint to approximately 90°, and POP was applied circumferentially from the groin to the ankle to restrict motion completely. The immobilization mimicked disuse osteoporosis, a well-established contributor to secondary bone loss in both animal and human studies. The fixation was allowed to harden for 10–15 minutes before returning the rat to its cage. To ensure consistency in immobilization throughout the study period, the casts were inspected daily for signs of loosening, cracking, or discomfort. If necessary, damaged casts were replaced under light ether anaesthesia. Body weight, limb colour, and mobility were monitored to detect circulatory or neurological complica-

tions. After each experimental endpoint (21, 28, and 35 days), the immobilization cast was carefully removed before sample collection.

Surgical procedure. A critical-sized bone defect (CSD) of 3 mm in diameter was surgically created in the mid-diaphyseal region of the femur for all experimental rats. The choice of a 3 mm defect was based on established preclinical models, which indicate that this dimension exceeds the natural bone healing capacity of rats within a typical observation period (4–6 weeks), thereby fulfilling the criteria for a CSD. Such a defect does not spontaneously heal without intervention, closely mimicking non-union or delayed union conditions observed in human long-bone fractures. This approach allows the study of bone remodelling dynamics and osteoporotic bone healing under controlled conditions. The defect also represents a clinically relevant scale of segmental bone loss, proportionally corresponding to defects of several centimetres in human femoral bone.

The surgical procedure was performed under general anaesthesia using a combination of ketamine hydrochloride and ether solution for induction and maintenance. After skin sterilization with povidone-iodine, a longitudinal incision was made over the lateral aspect of the femur to expose the bone. Using a low-speed microdrill (1.5 mm burr tip) under saline irrigation to prevent thermal necrosis, a circular 3 mm defect was created at the midshaft region. The defect site was treated according to the respective group protocols (e.g., synthetic bone graft, rh-BMP2, or combination treatment). The wound was irrigated with sterile saline and closed in layers using absorbable sutures. Postoperative care included a single dose of enrofloxacin (10 mg/kg, subcutaneously) and meloxicam (1 mg/kg, subcutaneously) to prevent infection and relieve pain.

Sample harvest and analysis. After the treatment period, rats were anesthetized using a combination of ketamine hydrochloride and ether solution to ensure pain relief. Once anesthetized, the animals were euthanized via an intraperitoneal injection of euthasol. The animals were then positioned in a supine position on a sterile surgical table, and the surgical site (the tibia) was cleaned with an antiseptic solution to prevent infection. A small incision was made over the tibia, and the surrounding soft tissue, including muscles and ligaments, was carefully dissected using sterile instruments. Once the tibia was exposed, a small section of the bone was excised using a sterile bone cutter or surgical scissors. The harvested tibial bone tissue was immediately placed in a sterile container on ice to preserve its integrity. The bone samples were then transferred to cold storage, typically in saline or PBS solution, to prevent enzymatic degradation. Following the preservation, the bone samples were cleaned of any remaining soft tissue and prepared for biochemical analysis. Bone fragments from the tibial diaphysis or metaphysis were processed for testing. TRACP-5b (Tartrate-resistant acid phosphatase 5b) and PINP (Procollagen Type 1 N-terminal propeptide) levels, markers for osteoclast and osteoblast activity, respectively, were measured using an ELISA Kit.

Statistical analysis

The collected data were organized, edited, and tabulated for analysis. A significance level of $p \leq 0.05$ was set, and a 95% confidence interval /CI) was used. One-way ANOVA was applied to compare the TRACP-5b and PINP levels across the different treatment groups and durations. Post-hoc tests were conducted to evaluate specific differences between the groups.

RESULTS

The data from the analysis of TRACP-5b and PINP levels from the 12 groups were first subjected to normality testing using the Shapiro-Wilk test and homogeneity testing using the Levene test to determine whether parametric or non-parametric statistical analysis should be applied. As the results of normality and homogeneity tests were met, an ANOVA was conducted for both TRACP-5b and PINP levels (Table 2). The Sum of Squares (SS) quantifies the total variation in the data. Specifically, the Sum of Squares Between Groups represents the variation due to the differences between the means of the 12 groups, while the Sum of Squares Within Groups reflects the variation within each group. The degrees of freedom (df) for between-groups was calculated as $k-1=12-1=11$, where k is the number of groups. The df within the groups was calculated as the total number of observations minus the number of groups, $n-k=48-12=36$, where n is the total number of data points. The df total is the total number of data points minus one, which is $n-1=48-1=47$.

Table 2. ANOVA test for procollagen Type 1 N-terminal propeptide (PINP) and tartrate-resistant acid phosphatase 5b (TRACP-5b) levels across treatment groups

Variable	Comparison	Sum of squares	df	Mean square	F	p
PINP	Between Groups	13042470.667	11	1185679.152	19.340	0.000
	Within Groups	2207088.000	36	61308.000		
	Total	5249558.667	47			
TRACP-5b	Between Groups	5.723	11	0.520	64.732	0.000
	Within Groups	0.289	36	0.008		
	Total	6.012	47			

Sum of squares, the total variation or dispersion in the data; df, degrees of freedom, refer to the number of independent values that can vary in the calculation without violating any given constraints; Mean square, an estimate of variance; F, the ratio of the variance between groups to the variance within groups, usually formulated as the mean square between groups divided by the mean square within groups;

The Mean Square (MS) is an estimate of the variance and it was calculated by dividing the Sum of Squares by the respective degrees of freedom. For example, MS Between Groups is the Sum of Squares Between Groups (13042470.667) divided by the df Between Groups (11), resulting in a Mean Square Between Groups (1185679.152) for PINP, and similarly for TRACP-5b. The F-value is the ratio of the variance between groups to the variance within groups, calculated as MS Between Groups / MS Within Groups. A larger F-value indicates that the variation between groups means is significantly greater than the variation within groups, suggesting a meaningful difference between the groups. For PINP, the F-value was 19.340, and for TRACP-5b 64.732, both with $p<0.05$, indicating that the differences between the treatment groups were statistically significant.

To specifically identify which groups exhibited statistically significant differences, a post hoc test was performed. Tukey HSD post hoc tests were chosen as they are most suitable for group comparisons.

The post hoc analysis comparing the TRACP-5b levels among

Table 3. Post Hoc tartrate-resistant acid phosphatase 5b (TRACP-5b)

Group for comparison	Comparing group	p	Group for comparison	Comparing group	p	Group for comparison	Comparing group	p	Group for comparison	Comparing group	p
C 21 days	C 28 days	0.900	G 21 days	C 21 days	0.001*	G+I 21 days	C 21 days	0.000*	G+I+CA 21 days	C 21 days	0.000*
	C 35 days	0.906		C 28 days	0.000*		C 28 days	0.000*		C 28 days	0.000*
	G 21 days	0.001*		C 35 days	0.000*		C 35 days	0.000*		C 35 days	0.000*
	G 28 days	0.000*		G 28 days	0.070		G 21 days	0.093		G 21 days	0.000*
	G 35 days	0.000*		G 35 days	0.012*		G 28 days	0.888		G 28 days	0.001*
	G+I 21 days	0.000*		G+I 21 days	0.093		G 35 days	0.360		G 35 days	0.006*
	G+I 28 days	0.000*		G+I 28 days	0.015*		G+I 28 days	0.413		G+I 21 days	0.000*
	G+I 35 days	0.000*		G+I 35 days	0.000*		G+I 35 days	0.001*		G+I 28 days	0.005*
	G+I+CA 21 days	0.000*		G+I+CA 21 days	0.000*		G+I+CA 21 days	0.000*		G+I 35 days	0.991
	G+I+CA 28 days	0.000*		G+I+CA 28 days	0.000*		G+I+CA 28 days	0.000*		G+I+CA 28 days	0.000*
	G+I+CA 35 days	0.000*		G+I+CA 35 days	0.000*		G+I+CA 35 days	0.000*		G+I+CA 35 days	0.000*
C 28 days	C 21 days	0.900	G 28 days	C 21 days	0.000*	G+I 28 days	C 21 days	0.000*	G+I+CA 28 days	C 21 days	0.000*
	C 35 days	0.994		C 28 days	0.000*		C 28 days	0.000*		C 28 days	0.000*
	G 21 days	0.000*		C 35 days	0.000*		C 35 days	0.000*		C 35 days	0.000*
	G 28 days	0.000*		G 21 days	0.070		G 21 days	0.015*		G 21 days	0.000*
	G 35 days	0.000*		G 35 days	0.438		G 28 days	0.497		G 28 days	0.000*
	G+I 21 days	0.000*		G+I 21 days	0.888		G 35 days	0.922		G 35 days	0.000*
	G+I 28 days	0.000*		G+I 28 days	0.497		G+I 21 days	0.413		G+I 21 days	0.000*
	G+I 35 days	0.000*		G+I 35 days	0.001*		G+I 35 days	0.005*		G+I 28 days	0.000*
	G+I+CA 28 days	0.000*		G+I+CA 21 days	0.001*		G+I+CA 21 days	0.005*		G+I 35 days	0.000*
	G+I+CA 35 days	0.000*		G+I+CA 28 days	0.000*		G+I+CA 28 days	0.000*		G+I+CA 21 days	0.000*
	G+I+CA 35 days	0.000*		G+I+CA 35 days	0.000*		G+I+CA 35 days	0.000*		G+I+CA 35 days	0.001*
C 35 days	C 21 days	0.906	G 35 days	C 21 days	0.000*	G+I 35 days	C 21 days	0.000*	G+I+CA 35 days	C 21 days	0.000*
	C 28 days	0.994		C 28 days	0.000*		C 28 days	0.000*		C 28 days	0.000*
	G 21 days	0.000*		C 35 days	0.000*		C 35 days	0.000*		C 35 days	0.000*
	G 28 days	0.000*		G 21 days	0.012*		G 21 days	0.000*		G 21 days	0.000*
	G 35 days	0.000*		G 28 days	0.438		G 28 days	0.001*		G 28 days	0.000*
	G+I 21 days	0.000*		G+I 21 days	0.360		G 35 days	0.006*		G 35 days	0.000*
	G+I 28 days	0.000*		G+I 28 days	0.922		G+I 21 days	0.001*		G+I 21 days	0.000*
	G+I 35 days	0.000*		G+I 35 days	0.006*		G+I 28 days	0.005*		G+I 28 days	0.000*
	G+I+CA 21 days	0.000*		G+I+CA 21 days	0.006*		G+I+CA 21 days	0.991		G+I 35 days	0.000*
	G+I+CA 28 days	0.000*		G+I+CA 28 days	0.000*		G+I+CA 28 days	0.000*		G+I+CA 21 days	0.000*
	G+I+CA 35 days	0.000*		G+I+CA 35 days	0.000*		G+I+CA 35 days	0.000*		G+I+CA 28 days	0.001*

*statistically significant difference;
C, Control; G: GnRH Agonist; I, immobilization; CA, low calcium intake

the treatment groups indicated that the combination of GnRH agonists, a low-calcium diet, and immobilization for 35 days yielded the most significant difference compared to the other groups (Table 3).

The post hoc results comparing each group to the others showed the combination of GnRH agonist, low calcium diet, and immobilization at 35 days showed the most significant difference when compared to others (Table 4).

DISCUSSION

The animal model used in this study combined the administration of a GnRH (Leuprolide acetate) agonist, a low-calcium diet, immobilization, and varying treatment durations. This combination is less commonly used, as most studies typically employ only one treatment per model. The administration of GnRH agonists can induce a hypogonadotropic-hypogonadal state, which accelerates bone loss (10). Additionally, adequate calcium intake is crucial for bone mass accumulation, and a calcium deficiency leads to reduced bone mass, often accompanied by a decrease in osteoblast numbers and an increase in osteoclast activity (11). Immobilization primarily results in bone loss in the hind limbs, which experience the most mechanical loading (12). The rate of bone loss is generally faster in cancellous bone than in cortical bone. Significant bone mass loss in the proximal and distal tibia metaphysis of this model typically occurs during the transition phase, between 14 and 30 days after immobilization (13).

Osteoclasts secrete TRACP into the circulation, making serum TRACP a valuable marker for osteoclast activity and bone resorption (14). In this study, TRACP-5b levels were significantly increased on days 21, 28, and 35. ANOVA analysis revealed a significant difference between the treatment groups in the TRACP-5b parameter. These findings align with previous studies on post-ovariectomy rats, which have shown increased TRACP-5b levels, indicating heightened osteoclast activity (15). The post hoc test revealed no significant difference in TRACP-5b levels between the GnRH agonist group on day 21 and the control group; however, a significant difference emerged on day 28. This is consistent with studies that show the effects of GnRH agonist treatment are typically observed from the second to the third month of administration (16-18). Statistically, no significant difference was observed between the GnRH agonist group and the group treated with GnRH agonist plus a low-calcium diet on day 21. However, immobilization significantly influenced the differ-

Table 4. Post Hoc procollagen type 1 N-terminal propeptide (PINP)

Group for comparison	Comparing group	p	Group for comparison	Comparing group	p	Group for comparison	Comparing group	p	Group for comparison	Comparing group	p
C 21 days	C 28 days	0.645	G 21 days	C 21 days	0.005*	G+I 21 days	C 21 days	0.000*	G+I+CA 21 days	C 21 days	0.000*
	C 35 days	0.581		C 28 days	0.015*		C 28 days	0.000*		C 28 days	0.000*
	G 21 days	0.005*		C 35 days	0.018*		C 35 days	0.000*		C 35 days	0.000*
	G 28 days	0.000*		G 28 days	0.039*		G 21 days	0.068		G 21 days	0.051
	G 35 days	0.000*		G 35 days	0.000*		G 28 days	0.790		G 28 days	0.896
	G+I 21 days	0.000*		G+I 21 days	0.068		G 35 days	0.012*		G 35 days	0.017*
	G+I 28 days	0.000*		G+I 28 days	0.034*		G+I 28 days	0.743		G+I 21 days	0.892
	G+I 35 days	0.000*		G+I 35 days	0.000*		G+I 35 days	0.013*		G+I 28 days	0.848
	G+I+CA 21 days	0.000*		G+I+CA 21 days	0.051		G+I+CA 21 days	0.892		G+I 35 days	0.019*
	G+I+CA 28 days	0.000*		G+I+CA 28 days	0.016*		G+I+CA 28 days	0.522		G+I+CA 28 days	0.613
	G+I+CA 35 days	0.000*		G+I+CA 35 days	0.000*		G+I+CA 35 days	0.000*		G+I+CA 35 days	0.000*
C 28 days	C 21 days	0.645	G 28 days	C 21 days	0.000*	G+I 28 days	C 21 days	0.000*	G+I+CA 28 days	C 21 days	0.000*
	C 35 days	0.927		C 28 days	0.000*		C 28 days	0.000*		C 28 days	0.000*
	G 21 days	0.015*		C 35 days	0.000*		C 35 days	0.000*		C 35 days	0.000*
	G 28 days	0.000*		G 21 days	0.039*		G 21 days	0.034*		G 21 days	0.016*
	G 35 days	0.000*		G 35 days	0.023*		G 28 days	0.951		G 28 days	0.707
	G+I 21 days	0.000*		G+I 21 days	0.790		G 35 days	0.027*		G 35 days	0.054
	G+I 28 days	0.000*		G+I 28 days	0.951		G+I 21 days	0.743		G+I 21 days	0.522
	G+I 35 days	0.000*		G+I 35 days	0.025*		G+I 35 days	0.029*		G+I 28 days	0.753
	G+I+CA 28 days	0.000*		G+I+CA 21 days	0.896		G+I+CA 21 days	0.848		G+I 35 days	0.058
	G+I+CA 35 days	0.000*		G+I+CA 28 days	0.707		G+I+CA 28 days	0.753		G+I+CA 21 days	0.613
	G+I+CA 35 days	0.000*		G+I+CA 35 days	0.000*		G+I+CA 35 days	0.000*		G+I+CA 35 days	0.000*
C 35 days	C 21 days	0.581	G 35 days	C 21 days	0.000*	G+I 35 days	C 21 days	0.000*	G+I+CA 35 days	C 21 days	0.000*
	C 28 days	0.927		C 28 days	0.000*		C 28 days	0.000*		C 28 days	0.000*
	G 21 days	0.018*		C 35 days	0.000*		C 35 days	0.000*		C 35 days	0.000*
	G 28 days	0.000*		G 21 days	0.000*		G 21 days	0.000*		G 21 days	0.000*
	G 35 days	0.000*		G 28 days	0.023*		G 28 days	0.025*		G 28 days	0.000*
	G+I 21 days	0.000*		G+I 21 days	0.012*		G 35 days	0.968		G 35 days	0.015*
	G+I 28 days	0.000*		G+I 28 days	0.027*		G+I 21 days	0.013*		G+I 21 days	0.000*
	G+I 35 days	0.000*		G+I 35 days	0.968		G+I 28 days	0.029*		G+I 28 days	0.000*
	G+I+CA 21 days	0.000*		G+I+CA 21 days	0.017*		G+I+CA 21 days	0.019*		G+I 35 days	0.014*
	G+I+CA 28 days	0.000*		G+I+CA 28 days	0.054		G+I+CA 28 days	0.058		G+I+CA 21 days	0.000*
	G+I+CA 35 days	0.000*		G+I+CA 35 days	0.015*		G+I+CA 35 days	0.014*		G+I+CA 28 days	0.000*

*statistically significant difference;
C, Control; G, GnRH Agonist; I, immobilization; CA, low calcium intake

ence, as it enhances the activation of the bone remodelling process and reduces osteoblast activity (19).

Procollagen-1 N-terminal peptide (PINP), a specific marker for type 1 collagen deposition, was consistently decreased in this study on days 21, 28, and 35. ANOVA analysis revealed a significant difference between the treatment groups in the PINP parameter ($p<0.05$). These results are consistent with previous research that demonstrated PINP as a predictor of bone loss, particularly in premenopausal women with systemic lupus erythematosus (20).

The biomarkers observed in this study indicate an increase in osteoclast activity and a decrease in osteoblast activity, suggesting an imbalance in bone remodelling. However, these findings should be further supported by X-ray and histopathological examinations. Dual-energy X-ray absorptiometry (DEXA) and histopathological analysis of rat bone tissue can help correlate laboratory biomarker titters with clinical imaging, providing a more comprehensive understanding of the bone loss process (21).

This study has several limitations. First, the use of a single animal model (Wistar rats) may not fully represent the complexity of human osteoporosis. The findings might not entirely translate to human conditions due to species differences. Additionally, the study duration (35 days) may be too short to observe the long-term effects of the combined treatment on bone density and microarchitecture. A small sample size may also limit the study's generalizability. Another limitation is that only biomarkers were used to assess bone turnover, which may not fully capture the structural changes occurring at the bone level. Radiological and histological confirmation would have provided a more comprehensive evaluation of the bone loss process.

Future studies should explore the long-term effects of GnRH agonists combined with a low-calcium diet and immobilization over a longer treatment period, extending the observation time to several months. Additionally, the use of different animal models, such as osteoporotic mice or large animal models could provide more insight into how these treatments affect various bone types and the structural integrity of bones. It would also be valuable to incorporate a combination of radiological imaging (such as DEXA and micro-CT scans) and histopathological examination to complement biomarker analysis, offering a more holistic view of bone remodelling. Furthermore, future research could investigate the effects of different dosages and combinations of treatments to identify the most effective regimen for inducing secondary osteoporosis. Ultimately, investigating the molecular

mechanisms underlying the changes in osteoclast and osteoblast activity in response to this combined treatment could provide deeper insights into the pathophysiology of osteoporosis.

In conclusion, this study successfully demonstrated that the combination of GnRH agonists (Leuprolide acetate), a low-calcium diet, and immobilization can effectively induce secondary osteoporosis in Wistar rats, as evidenced by significant changes in TRACP-5b and PINP levels. The results indicate an imbalance in bone remodelling, characterized by increased osteoclast activity and decreased osteoblast function. The findings suggest that this combined treatment approach provides a reliable and alternative method for creating experimental models of secondary osteoporosis. Future research should further investigate the long-term effects and molecular mechanisms of this model to understand its potential applications in osteoporosis studies better.

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Conflicts of interest: None to declare.

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