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RESEARCH ARTICLE

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SARM Rad140 Increases Osteoblasts, Muscle Fiber Size, Myonuclei, and Reduces Osteoclasts in Orchidectomized Wistar Rats

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Background: Orchidectomy is a surgical androgen deprivation therapy (ADT) for prostate cancer patients to achieve castrate testosterone levels. Selective androgen receptor modulators (SARMs) are used to mitigate the adverse effects of ADT, including elevated risk of osteoporosis, and reduced skeletal muscle mass. Rad140 is a novel SARMs that has high affinity for the androgen receptors. This study was conducted to determine the effects of SARM Rad140 on the number of osteoblasts, osteoclasts, muscle fiber cross-sectional area (CSA), muscle size, and number of myonuclei in rats underwent orchidectomy.

Materials and methods: An experimental study was conducted using a randomized post-test only control group design. Following orchidectomy, SARM Rad140 was administered orally for six weeks at various doses. Osteoblasts, osteoclasts, muscle fiber CSA, muscle size, and number of myonuclei were measured. Quantitative analysis was performed using one-way ANOVA.

Results: There were significant differences in the effects of SARM Rad140 at different doses on osteoblast and osteoclast cells. At higher doses, the osteoblast cell counts in rats tended to increase, while the osteoclast counts tended to decline. The treatment group also showed significant results in the CSA of the gastrocnemius muscle fibers, as well as in the number of myonuclei of the gastrocnemius muscle.

Conclusion: SARM Rad140 significantly increased the number of osteoblasts, muscle fiber CSA, and gastrocnemius muscle myonuclei, while decreasing osteoclasts. SARM Rad140 is a promising therapy for osteoporosis and muscle weakening due to ADT.

Keywords: *SARM Rad140, orchidectomy, osteoblasts, osteoclasts, muscle fiber cross sectional area, myonuclei*

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Introduction

Prostate cancer is the second most common disease among males, with an expected 1.4 million cases worldwide in 2020.[1] In Indonesia, three educational hospitals (in Jakarta, Surabaya, and Bandung) have treated 1,102 prostate cancer patients over the past eight years, with an average patient age of 67.18 years. Androgen deprivation therapy (ADT) includes surgical and medical castration methods to reduce testosterone and dihydrotestosterone levels.[2] Both surgical and medical castration methods are equally effective in achieving testosterone levels below 50 ng/dL while controlling cancer. Orchidectomy, a surgical ADT, can be performed using bilateral or subscapular orchidectomy procedures.[3]

ADT can cause hot flushes, depression, drowsiness, increased visceral and abdominal fat mass, elevated cholesterol and triglyceride levels, weight gain, insulin resistance, sarcopenia, loss of bone mineral density (increasing the risk of fractures), and a decrease in skeletal muscle mass.[4] Selective androgen receptor modulators (SARMs) are ligands for androgen receptors (AR) that enhance tissue-specific androgen signaling.[5] SARMs can selectively stimulate androgenic signaling in certain tissues with AR, regulating osteoblasts, osteoclasts, and muscle growth. Although the effects of SARMs have been investigated in mice undergoing orchidectomy, previous studies indicate that supplementation with Rad140 can increase muscle strength and mass in experiments using in vivo eccentric contractions to train mice's dorsiflexor muscles and detect changes in tetanic isometric torque.[5,6]

Focusing on their adverse effects, benefits, and public interest in their use, data on SARMs' effects on osteoblasts, osteoclasts, and muscle mass, particularly histologically, remain limited. SARM Rad140 demonstrated strong stability ($t1/2 > 2$ h) in incubations with rat, monkey, and human microsomes, as well as good bioavailability (65-75% in rats and monkeys). Rad140 showed strong affinity for the androgen receptor and good selectivity over other steroid hormone nuclear receptors, being fully anabolic on muscle.[7] SARM Rad140 was chosen for this study to manage the negative effects of ADT therapy. Thus, this study was conducted to determine the effect of SARM Rad140 on the number of osteoblasts, osteoclasts, muscle fiber cross-sectional area (CSA), muscle size, and amount of myonuclei in gastrocnemius muscle in patients who underwent orchidectomy.

Materials and methods

Sample Recruitment

Sampling was conducted using a simple random sampling technique, whereby samples were selected from the population randomly, without considering the population's characteristics. The sample size for each group was calculated using the Federer formula $(n-1)(t-1) \geq 15$, where n represents the sample size for each intervention and t is the number of interventions, with an expected dropout of 10%, the final sample size for each group was determined to be five samples.[8]

Male Wistar rats (*Rattus norvegicus*) were acclimatized in the Pharmacology Laboratory of the Faculty of Medicine, Universitas Brawijaya, for seven days. Inclusion criteria required that the rats be male, white Wistar rats aged three months, healthy, and weighing between 200 and 250 grams, all obtained from the same breeding center and fed the same diet.

There were seven groups in total, consist of the control groups and the experimental groups. The control groups included were the negative control group (untreated) which do not receive orchidectomy procedure, and the positive control group (Sham) which received orchidectomy procedure. The experimental groups consisted of orchidectomized rats administered with SARM Rad140 (Ellipses Pharma, London, UK) orally at different doses of 0.03 mg/kg Rad140 (T1), 0.1 mg/kg Rad140 (T2), 1 mg/kg Rad140 (T3), 3 mg/kg Rad140 (T4), and 10 mg/kg Rad140 (T5) mg/kg, suspended in 0.5% methylcellulose and given over a period of six weeks.9 Each dose of SARM Rad140 suspension was made in the same appearance (color, thickness, volume, and jar) to minimize bias, and the administration was controlled by one controller, in addition to the main researchers. This study protocol was approved by the Health Research Ethics Commission of Dr. Saiful Anwar General Hospital, with approval number 400/307/K.3/102.7/2022.

Orchidectomy Procedure

Orchidectomy was performed under general anesthesia. The rats were deeply anesthetized using ether and positioned on a surgical board in a dorsal recumbent position. A scalpel was employed to make a 1 cm incision in the raphe of the scrotum, followed by the dissection of the cremaster muscle. The testicular contents were exposed by gently removing the testicular fat pad with blunt, sterile forceps. The cauda

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epididymis, caput epididymis, vas deferens, and testicular blood vessels were carefully dissected. The vas deferens and blood vessels were ligated to minimize bleeding following testicular removal. The epididymis and caput epididymis were separated from the testis, and the testis was excised. The incision was closed by suturing the skin.[10]

Buprenorphine (0.05 mg/kg) was administered subcutaneously in the dorsal neck of the rat for analgesia. Fluid lost during the surgery was replaced with warm normal saline, given subcutaneously or intraperitoneally. The rats were closely monitored for 2-4 hours until they recovered from anesthesia. Once healed, approximately 24 hours post-surgery, they were returned to their cage with the other rats.[10] At the end of the sixth week, histological preparations of the rats' bone and muscle were examined to determine the number of osteoblasts, osteoclasts, muscle fiber CSA, myonuclei, and gastrocnemius muscle mass.

Bone Histological Preparation and Staining

Rats' femur bones were decalcified by soaking in 10% neutral buffered formaldehyde (NBF) for 24 hours. The samples were rinsed with flowing tap water and incubated in Surgipath Decalcifier I (Leica Biosystem, Deer Park, USA) until fully submerged for 7 days. Decalcification was conducted at room temperature until the bone could be easily punctured with a needle. Following rinsing in running tap water, the decalcified bones underwent regular dehydration.

The samples were processed in a tissue processor using graded ethanol concentrations (70%, 80%, 95%, and 3×100%), immersed in xylene three times, and embedded in paraffin at 60°C for 40 minutes each time. Specimens were then cooled in ice water overnight before being cut into 5 µm thick sections using a Leica microtome, observed in transverse sections, and mounted on polylysine slides. After drying in an oven at 45°C for at least 5 hours, hematoxylin and eosin (HE) staining was performed following deparaffinization in xylene and rehydration in graded ethanol (100-50%).

Osteoblast counting was based on their characteristics; these cells, typically cuboidal or columnar, are found on the surface of the bone matrix, each possessing a spherical nucleus and basophilic cytoplasm. In contrast, osteoclasts are multinucleated and often located in lacunae or on the bone surface.

Muscle Histological Preparation and Staining

Gastrocnemius muscle tissue was fixed in 10% formalin or 10% formalin buffer for at least 7 hours. The tissue was then cut into approximately 2-3 mm thick sections, cleansed, and weighed. Tissue processing was conducted for 90 minutes using an Automatic Tissue Tex Processor, followed by paraffin embedding. The samples were sliced into 3-5 microns thick sections using a microtome. HE staining was performed for histological observation of the gastrocnemius muscle, including assessment of muscle fiber cross-sectional area (CSA) and myonuclei.

These variables were examined using an Olympus CX23 light microscope at a magnification of 400x with a field of view of 10x. Muscle fiber CSA was measured as the area of the cross-section of a muscle, taken perpendicular to its fibers, typically at the largest point visible under the microscope. Myonuclei, which are the nuclei located centrally within muscle cells, were identified based on their distinctive thicker appearance compared to muscle fibers. The number of myonuclei was quantified accordingly. Muscle mass was determined by measuring the wet weight of the skeletal muscle. The gastrocnemius muscle was excised, cleaned, and weighed on an analytical scale.

Results

SARM Rad140 Increased Osteoblasts and Decreased Osteoclasts

Osteoclasts, which are identified with their multinucleated and giant cell morphology, were observed to decrease in number with increasing doses of Rad140, as showed with the red arrows (Figure 1). In contrast, osteoblasts, which are characterized with spherical nuclei located in basal cells and typically containing one to three nucleoli, appeared to increase in number, as showed with the blue arrows. In Untreated and Sham groups, both osteoblasts and osteoclasts were present in moderate amounts. However, in the Treated groups (T1, T2, T3, T4, and T5), Rad140 administration led to a notable increase in osteoblast activity, coupled with a reduction in osteoclastic presence.

The Sham group showed a marked decrease in osteoblasts and an increase in osteoclasts (Table 1). As the SARM Rad140 was administered at increasing doses, a clear dose-dependent effect emerged (ANOVA, p=0.000). At the lowest dose (T1 group), the number of osteoblasts increased, although the number of osteoclasts remained relatively high. As the dose increased to T2 and T3 groups, the number of osteoblasts continued to increase, while the number of osteoclasts began to decline slightly. The most pronounced effects were observed at higher doses. At T4 group, the number of osteoblasts increased, and the number

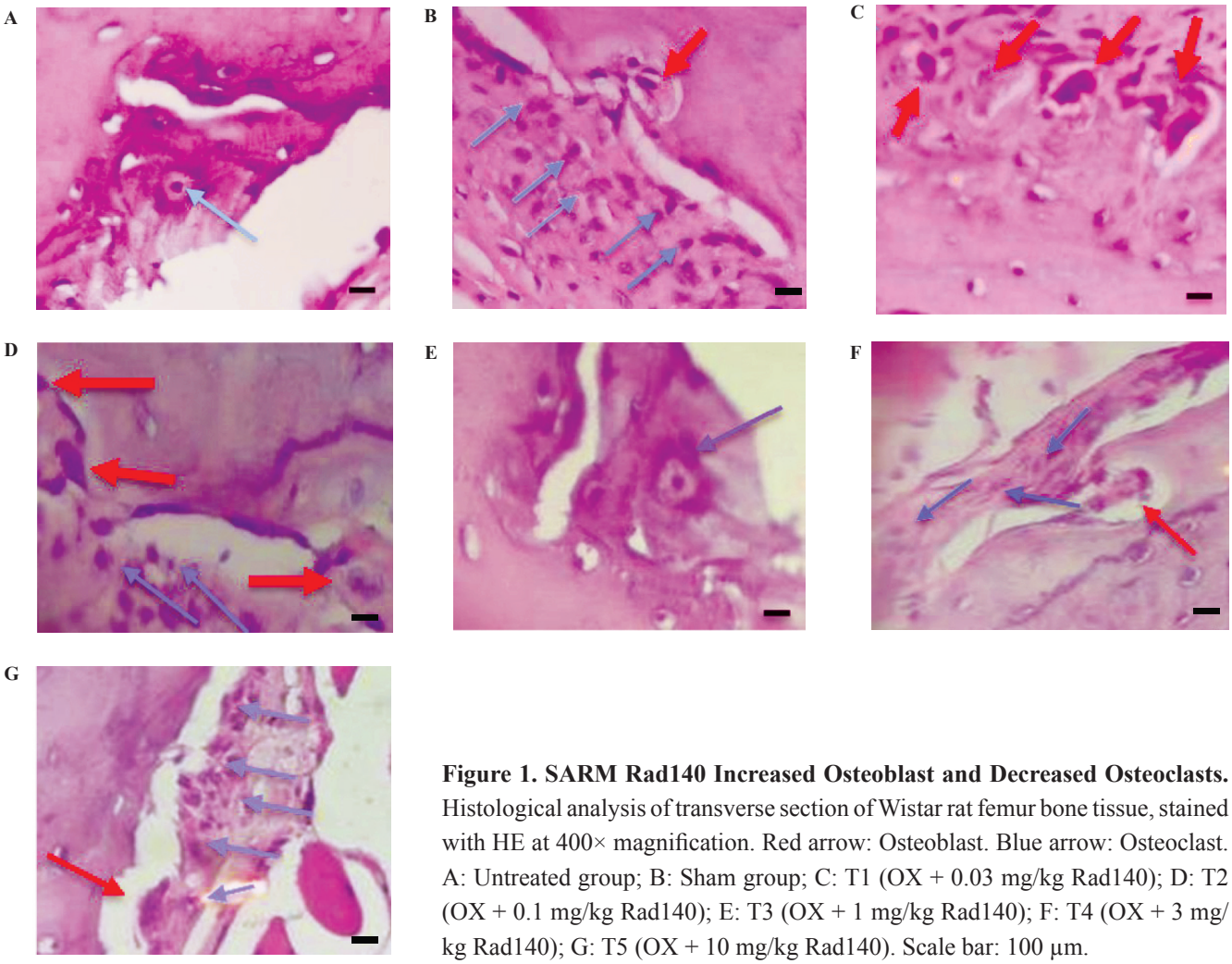

Figure 1. SARM Rad140 Increased Osteoblast and Decreased Osteoclasts. Histological analysis of transverse section of Wistar rat femur bone tissue, stained with HE at 400× magnification. Red arrow: Osteoblast. Blue arrow: Osteoclast. A: Untreated group; B: Sham group; C: T1 (OX + 0.03 mg/kg Rad140); D: T2 (OX + 0.1 mg/kg Rad140); E: T3 (OX + 1 mg/kg Rad140); F: T4 (OX + 3 mg/kg Rad140); G: T5 (OX + 10 mg/kg Rad140). Scale bar: 100 µm.

of osteoclasts decreased significantly (Tukey's post hoc test, p=0.000). Similarly, the T5 group (the highest dose) showed a further increase in osteoblasts, with osteoclasts reduced.

SARM Rad140 Increased Muscle Fiber CSA

In the Untreated and Sham groups, the CSA of muscle fibers, showed with the yellow outlines, appeared relatively small (Figure 2). However, as the doses of Rad140 increased (T1-T5 groups), a progressive enlargement of the CSA was evident, particularly in T4 and T5 groups.

The muscle fiber CSA of the gastrocnemius muscles in the Untreated group (189.15±56.79 mm^2) had the highest CSA and significantly different (p=0.000) than the ones in the Sham group (140.4±79.17 mm^2) (Figure 3). The CSA increased in a dose-dependent manner (ANOVA, p=0.000) with SARM Rad140 administration. The CSA in the T1

Table 1. SARM Rad140 increased osteoblast and decreased osteoclasts in a dose-dependent manner.

Cell	Number of Cells (per 10x Field of View)						
	Untreated	Sham	T1	T2	T3	T4	T5
Osteoblasts	89±2.58	13.5±1.91	26±3.65	37±2.58	50.5±7	79.5±5.97	82±3.65
Osteoclasts	7±1.63	22.5±1.29	22±1.83	19.25±1.36	18.5±2.38	10.5±1.29	9.25±1.71

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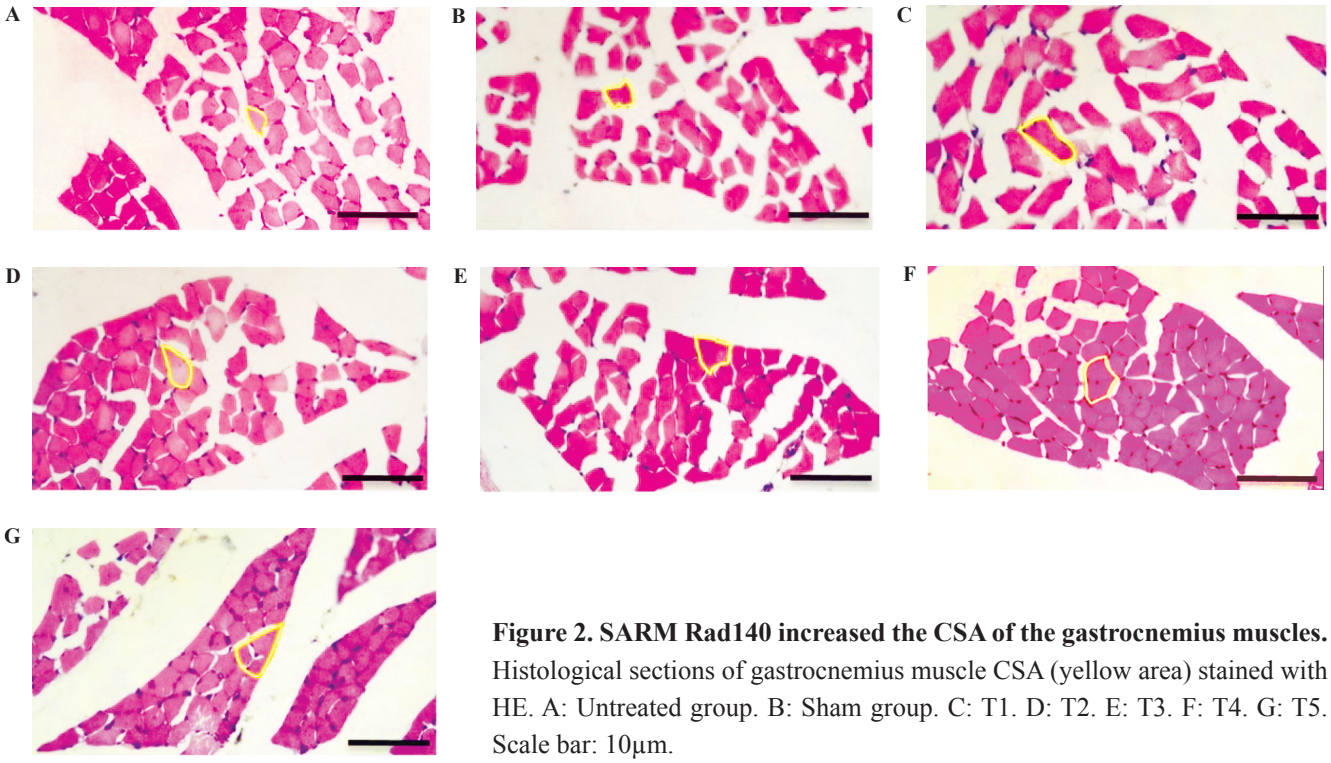

Figure 2. SARM Rad140 increased the CSA of the gastrocnemius muscles. Histological sections of gastrocnemius muscle CSA (yellow area) stained with HE. A: Untreated group. B: Sham group. C: T1. D: T2. E: T3. F: T4. G: T5. Scale bar: 10µm.

(149.65±49.33 mm^2, p=0.440) and T2 (154.58±69.03 mm^2, p=0.072) groups did not significantly differ than the ones in the Sham group. In contrast, the CSA in the T3 (164.03±92.81 mm^2, p=0.001), T4 (174.9±35.82 mm^2, p=0.000) and T5 (180.63±20.65 mm^2, p=0.000) groups showed significant increase than the ones in the Sham group.

SARM Rad140 Increased Gastrocnemius Muscle Myonuclei

In the Untreated and Sham groups, the number of myonuclei appeared relatively low (Figure 4). However, in the Rad140-treated groups, there was a dose-dependent increase in the number of myonuclei, as showed with the yellow arrows.

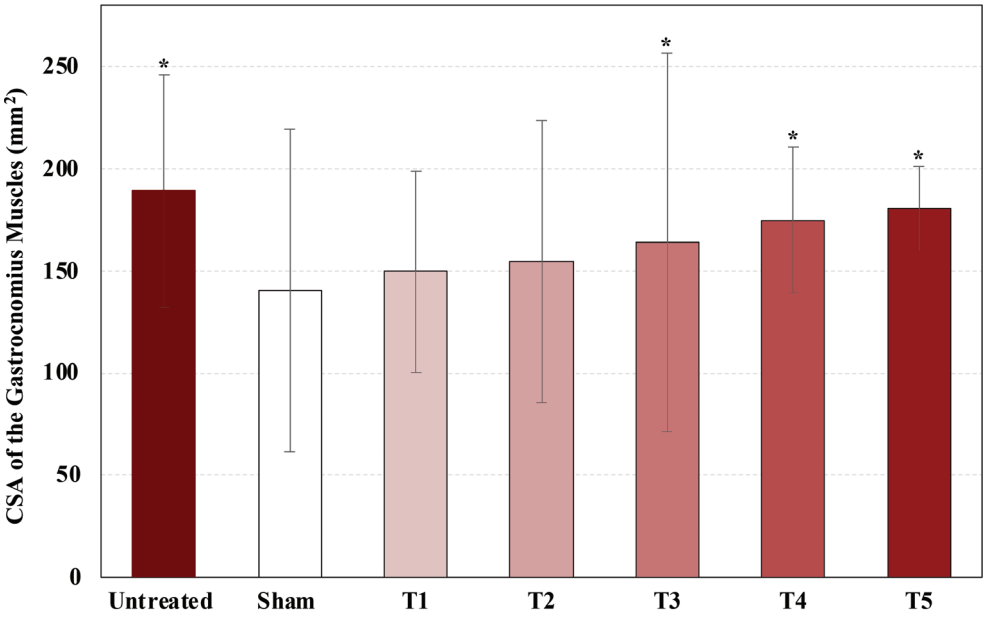

Figure 3. SARM Rad140 increased the CSA of the gastrocnemius muscles in a dose-dependent manner. The data are presented as mean±standard deviation (n=4). *Statistical significance (p<0.05) was determined using Tukey's post hoc test when compared with the Sham group.

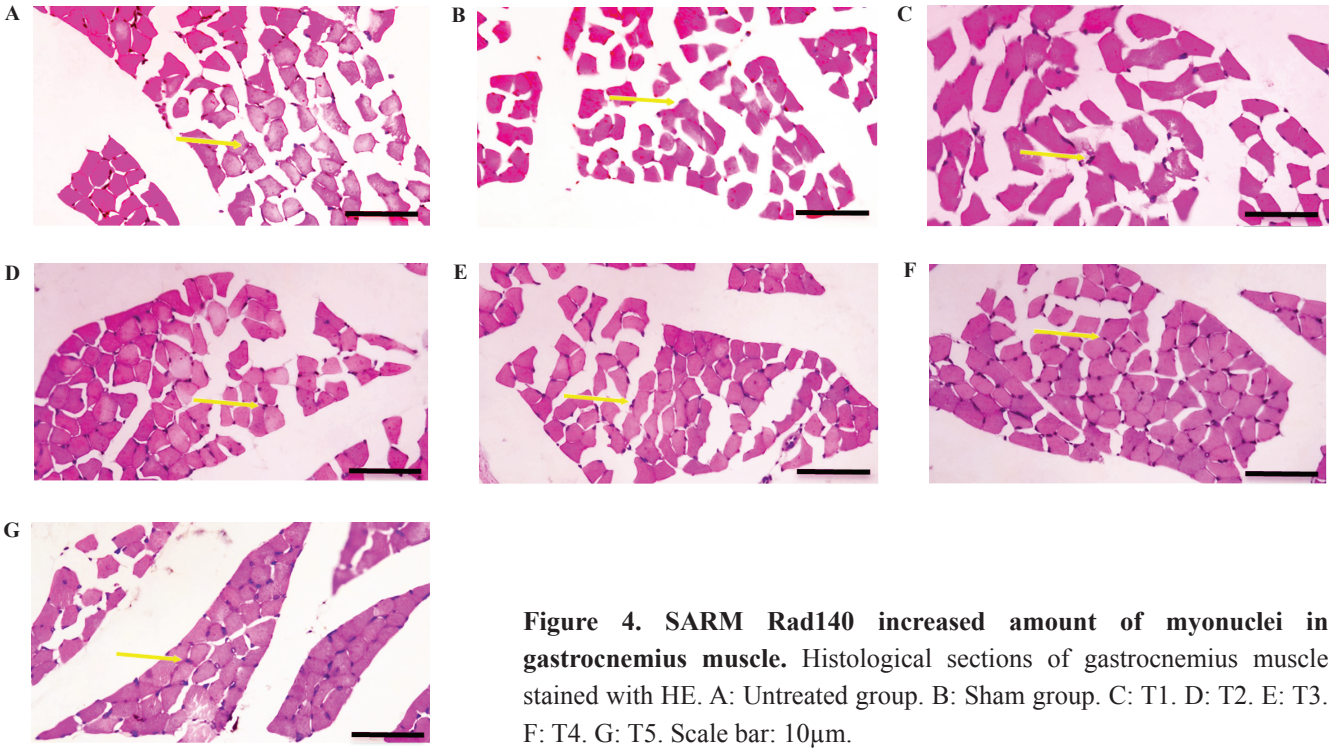

Figure 4. SARM Rad140 increased amount of myonuclei in gastrocnemius muscle. Histological sections of gastrocnemius muscle stained with HE. A: Untreated group. B: Sham group. C: T1. D: T2. E: T3. F: T4. G: T5. Scale bar: 10µm.

The gastrocnemius muscles myonuclei amount in the Untreated group (465±4.64) had the highest amount of myonuclei, while the Sham group showed a marked reduction (265±5.43) (Figure 5). The myonuclei amount increased in a dose-dependent manner with SARM Rad140 administration. The myonuclei amout in the T1 (319±6.7, p=0.065) and T2 (336±7.93, p=0.287) groups did not significantly differ than the ones in the Sham group. In

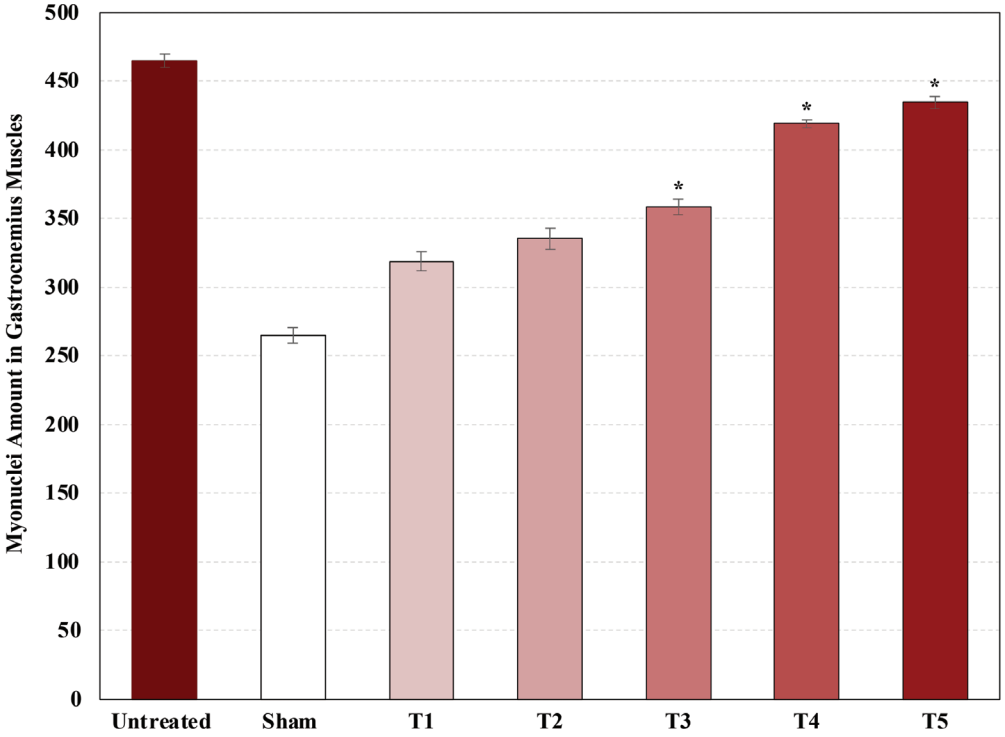

Figure 5. SARM Rad140 increased amount of myonuclei in gastrocnemius muscle in a dose-dependent manner. The data are presented as mean±standard deviation (n=4). *Statistical significance ($p<0.05$) was determined using Tukey's post hoc test when compared with the Sham group.

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contrast, the T3 (358.75±5.56, p=0.005), T4 (419.25±2.87, p=0.001) and T5 (434.75±4.65, p=0.001) groups showed significant increase than the ones in the Sham group.

Discussion

This study showed that the number of rat osteoblast cells tended to increase while the number of osteoclast cells tended to decrease when given higher doses of SARM Rad140. Reductions in androgen levels have been implicated in the loss of muscle mass, strength, and bone density, which can lead to osteoporosis.[7] SARMs act as selective agonists in androgen-responsive tissues, including bone and muscle. They influence various bone cells, such as osteoblasts, which express androgen receptors (AR) and are responsive to testosterone.[11,12] Previous research indicated that androgens can affect osteoblast markers like osteocalcin, collagen type 1, and alkaline phosphatase, impacting bone matrix mineralization in a context-dependent manner.[13]

The development of osteoclasts following orchidectomy is most likely caused by testosterone deprivation. Orchidectomy promotes the formation of osteoblast precursors that release receptor activator of nuclear factor kappa-B ligand (RANKL), which enhances osteoclast proliferation and activation.[14] A previous study of femur bones from orchidectomized rats revealed the presence of fractured bone matrix and enhanced activation of multinucleated osteoclasts responsible for bone disintegration and resorption.[14] Another study also showed an increase in the amount of osteoclasts, tartrate-resistant acid phosphatase (TRAP) positive osteoclast surface, receptor activator of nuclear factor kappa-B ligand-osteoprotegerin ratio (RANKL/OPG ratio), and RANKL gene expression levels in treatment group using tibia bone from orchidectomized rats.[15] This suggests that SARM Rad140 may counteract these effects by selectively stimulating anabolic pathways in muscle and bone, which is crucial for developing therapeutic strategies for osteoporosis.[16] Furthermore, by altering the transcriptional response of the SARM-AR complex, SARMs are predicted to restrict the process of decreasing AR binding, which can lead to osteoporosis. In addition, treating cells with SARM S-4 dramatically reduced the amount of generated multinucleated osteoclasts while also preventing bone loss and lowering body fat in ovariectomized rats via RANKL and granulocyte-macrophage colony-stimulating factor (GM-CSF) activation.[17]

This study found that muscle fiber CSA measurements were lower in positive controls due to the presence of androgen insufficiency caused by orchidectomy. Orchidectomy drastically lowered male rats' muscle weight and strength. This effect was inversely related to adding dihydrotestosterone (DHT) therapy.[18] Testosterone promotes satellite cell replication and activation, as well as the quantity of myonuclei, and has an effect on protein metabolism. The androgen-dependent myogenic effects on cellular differentiation, proliferation, and muscle protein turnover are regulated by several signaling pathways.[19] When androgens interact with the androgen receptor, catenin migrates to the nucleus of mesenchymal pluripotent cells. This leads to myogenic differentiation through follistatin signaling and suppresses transforming growth factor-β (TGF- β).[20] The changes in muscle fiber CSA observed in this study align with other research showing that SARMs can enhance muscle growth following orchidectomy.[21] SARMs medication enhanced the muscle fiber CSA in female rats who were ovariectomized, in addition to male experimental animals.[22,23]

SARMs have been designed to induce anabolic effects on muscles and bones while avoiding the dose-limiting androgenic effects of testosterone. An increase in myonuclei is one evidence of SARMs therapy's effectiveness in improving muscle mass.[23] These finding were in line with prior research, which found that administering SARMs to rats in the orchidectomy model greatly increased the amount of myonuclei, or cell nuclei. The presence of androgen receptors causes satellite cells to proliferate, differentiate, and fuse, forming new myofibers that are subsequently integrated into existing muscle fibers by contributing their nuclei, resulting in skeletal muscle hypertrophy.[24,25]

Bilateral orchidectomy reduced testosterone levels, which in turn reduced muscle mass. SARMs are administered to alleviate these negative effects.[24] In this study, various doses of SARM Rad140 were shown to increase gastrocnemius muscle mass. These findings are also consistent with prior studies in which SARMs were demonstrated to increase muscle growth in males separated into groups receiving small, gradually increasing doses and placebo-controlled groups for 21 days. The SARMs were well tolerated, with significant dose-proportional gains in muscle mass.[26,27]

However, this study has limitations, including the lack of preliminary research on the optimal duration of SARM Rad140 administration, a short study duration, absence of

female subjects, and variability in individual responses to Rad140. Future research should focus on these aspects to better understand the long-term effects of SARMs.

Conclusion

SARM Rad140 significantly increased the number of osteoblasts, muscle fiber CSA, and gastrocnemius muscle myonuclei, as well as decreased the number of osteoclasts. SARM Rad140 may offer potential benefits as a therapy for osteoporosis and muscle weakening due to ADT, as the treatment has been shown to increase bone density.

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Authors' Contributions

BD, TNB, PNT, and RARKD contributed to the conceptualization, methodology, writing of the original draft, investigation, supervision, and validation of the research. DMF and AAE also played key roles in conceptualization, methodology, writing the original draft, investigation, and data curation. FKD focused on methodology, writing the original draft, investigation, and data curation. Additionally, AFR was responsible for visualization and project administration.

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