EXPERIMENTAL / BASIC SCIENCE RESEARCH

acta orthopaedica belgica, 2026, 92, online-only

doi.org/10.52628/92.2.15471

Reduced Serum Klotho Levels in Disuse-Induced Skeletal Muscle Atrophy: An Experimental Study Related to Orthopedic Immobilization

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ABSTRACT

Klotho is an anti-aging protein involved in phosphate homeostasis, oxidative stress regulation, and tissue regeneration. Although circulating Klotho levels decline with chronological aging, its response to acute disuse-induced skeletal muscle alterations remains unclear. In this experimental study, sixteen male Wistar rats (3 months old) were randomly assigned to a control group (n = 8) or an intermittent hindlimb unloading group (3 h/day for 15 consecutive days) (n = 8). Skeletal muscle morphology was evaluated in the extensor digitorum longus (EDL) and soleus muscles using hematoxylin–eosin staining, and muscle fiber cross-sectional area (CSA) was quantified with ImageJ software. Interstitial connective tissue changes were qualitatively assessed using Masson's trichrome staining, and serum Klotho concentrations were measured using enzyme-linked immunosorbent assay (ELISA). Intermittent unloading induced histological features consistent with early disuse-associated muscle remodeling in both EDL and soleus muscles, accompanied by a modest reduction in muscle fiber CSA compared with controls. Serum Klotho levels were significantly lower in the unloading group (7.44 ± 0.54 vs 8.21 ± 0.81, p = 0.045), and a moderate positive correlation was observed between circulating Klotho concentrations and mean muscle fiber CSA (r = 0.58, p = 0.02). Mild interstitial connective tissue expansion was qualitatively noted in the soleus muscle following unloading. These findings suggest that short-term intermittent hindlimb unloading in young adult rats is associated with reduced circulating Klotho levels and mild skeletal muscle remodeling. Serum Klotho may represent a candidate biomarker of early disuse-associated muscle alterations; however, further studies are required to confirm its clinical applicability. These results may have potential implications for orthopedic conditions characterized by immobilization, including fracture treatment, casting, and postoperative rehabilitation.

Keywords: Klotho, hindlimb unloading, skeletal muscle atrophy, orthopedic immobilization, rat model.

INTRODUCTION

The anti-aging gene klotho was first identified in 1997 and named after the mythological Greek goddess who spins the thread of life[1]. Experimental studies have demonstrated that klotho-deficient mice develop a constellation of phenotypes resembling accelerated aging, including reduced lifespan, skin atrophy, osteoporosis, sarcopenia, atherosclerosis, infertility, and emphysema[1]. In contrast, overexpression of klotho has been shown to extend lifespan by approximately 20–30% in mice, highlighting its central role in longevity and tissue homeostasis[2].

Klotho is a type I transmembrane protein predominantly expressed in the distal renal tubules and, to a lesser extent, in other tissues such as the brain and liver, where it functions as an important metabolic and anti-aging regulator[1]. Although the kidney represents the principal source of circulating klotho, expression has also been reported in the parathyroid gland and choroid plexus[3]. The extracellular domain of membrane-bound klotho is cleaved by a disintegrin and metalloproteinase (ADAM), releasing soluble klotho into the renal interstitium and subsequently into the systemic circulation[4,5]. Circulating klotho interacts with several membrane-associated receptors, including those involved in transforming growth factor-β (TGF-β) and insulin-like growth factor (IGF) signalling pathways[2,6]. Through inhibition of IGF signalling, klotho has been reported to induce the expression of antioxidant

enzymes such as superoxide dismutase, while potentially suppressing Wnt and TGF-β signalling pathways to limit fibrotic processes[2,7].

Soluble klotho is detectable in blood, urine, and cerebrospinal fluid[8] and plays an essential role in calcium and phosphate homeostasis. In the kidney, calcium reabsorption in the distal tubule is mediated primarily by transient receptor potential vanilloid type 5 and type 6 (TRPV5 and TRPV6) channels[9-11]. The secreted form of klotho enhances the activity of these channels, and klotho-deficient mice consequently exhibit increased urinary calcium excretion[12,13]. This renal calcium loss may secondarily promote increased calcium mobilization from bone, thereby linking klotho deficiency to skeletal fragility.

Klotho exists in two major forms: circulating α-klotho (c-α-klotho) and membrane-bound α-klotho (m-α-klotho). Membrane-bound klotho acts as a co-receptor for fibroblast growth factor-23 (FGF-23), a hormone produced by osteocytes and osteoblasts, and together they exert a phosphaturic effect in the renal tubular epithelium[8,14,15]. Animals lacking either FGF-23 or α-klotho display a phenotype resembling accelerated aging and shortened lifespan[1,15]. Dietary phosphate restriction has been shown to attenuate this aging-like phenotype and prolong survival in α-klotho–deficient mice, suggesting that phosphate dysregulation plays a key role in klotho-mediated aging processes[16]. However, the molecular mechanisms through which circulating α-klotho exerts its systemic anti-aging effects remain incompletely understood.

Emerging evidence indicates that circulating α-klotho may also influence skeletal muscle homeostasis. The c-α-klotho protein has been shown to inhibit TGF-β1 signalling by blocking the type II serine/threonine kinase receptor (TbRII), thereby attenuating fibrosis[6]. In addition to TGF-β1, c-α-klotho appears to suppress other muscle-wasting TGF-β family members, including myostatin, which is a well-established negative regulator of muscle mass[17]. Through these shared signalling pathways, klotho may exert protective effects against muscle wasting and degeneration.

Sarcopenia is traditionally defined as an age-related condition characterized by progressive loss of skeletal muscle mass and strength[18]. Skeletal muscle constitutes more than 40% of total body mass in young adults; however, muscle mass declines by approximately 0.1–0.5% per year after the age of 30[19]. Epidemiological studies estimate that sarcopenia affects nearly 40% of individuals over 80 years of age and approximately 25% of those under 70[20]. The clinical consequences of sarcopenia include reduced mobility, increased risk of falls and fractures, loss of independence, and increased mortality in both humans and experimental animals[21,22].

Importantly, experimental studies in mice have demonstrated that age-related conditions such as osteopenia and sarcopenia are closely associated with disrupted klotho expression[23]. In humans, low circulating klotho levels have been linked to reduced bone mineral density and diminished grip strength, further supporting a role for klotho in musculoskeletal health[24,25]. While the relationship between klotho and age-related muscle loss has been increasingly explored, the effects of acute muscle disuse on circulating klotho levels remain poorly defined.

Disuse-induced skeletal muscle atrophy resulting from immobilization or mechanical unloading represents a clinically relevant musculoskeletal problem encountered in prolonged bed rest, hospitalization, and orthopedic immobilization, such as fracture casting, ligament injuries, and postoperative inactivity. Although disuse atrophy has been proposed to contribute to sarcopenia-like phenotypes, it remains unclear whether short-term muscle disuse independently affects circulating klotho levels. In orthopaedic clinical practice, such as during the recovery phase of major fracture fixations or following joint arthroplasty, prolonged immobilization is often unavoidable. Understanding the early biological markers of this process is crucial for optimizing rehabilitation outcomes.

Therefore, the aim of the present study was to investigate serum klotho levels in a rat model of disuse-induced skeletal muscle atrophy and to examine the potential association between circulating klotho and muscle morphological changes. By addressing this gap, the present study seeks to provide experimental evidence suggesting a role for klotho in early disuse-related muscle degeneration, potentially offering new insights for orthopaedic rehabilitation strategies.

METHODS

Animals and Experimental Design

The study protocol was approved by the Laboratory Animal Care and Use Committee of Fırat University Faculty of Medicine (approval date: 17 July 2023; approval number: 17217). Sixteen male Wistar albino rats (Rattus norvegicus), aged 3 months and weighing 220 ± 20 g, were included. Animals were randomly assigned using a computer-generated randomization method to either a control group (n = 8) or a disuse group (n = 8). A schematic overview of the experimental design is presented in Figure 1.

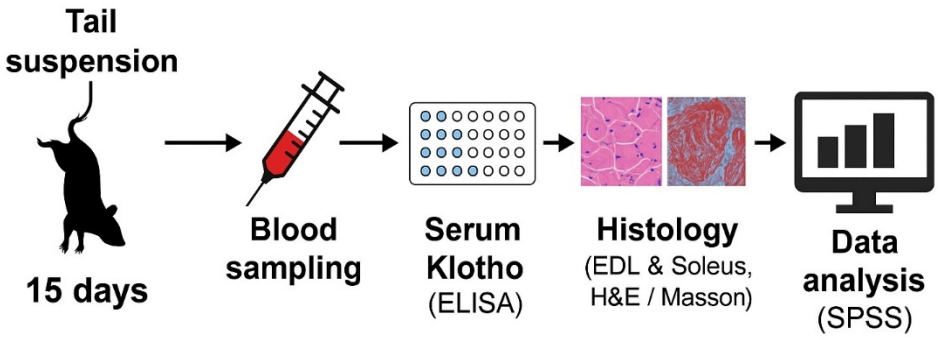

Fig. 1 — Experimental workflow of the study.
Rats were allocated to control or intermittent hindlimb unloading groups (3 h/day for 15 consecutive days). At the end of the intervention, blood samples were collected for serum Klotho measurement by ELISA. Extensor digitorum longus (EDL) and soleus muscles were harvested for histological and morphometric analyses.

Rats were housed four per cage under standard laboratory conditions (12:12 h light–dark cycle, temperature 22 ± 2 °C, humidity 50 ± 5%) with ad libitum access to standard laboratory chow and water.

Disuse was induced using an intermittent hindlimb unloading (HU) protocol adapted from previously described unloading models[26]. Rats in the disuse group were anesthetized briefly with ketamine (75–90 mg/kg) and xylazine (5–10 mg/kg, intraperitoneal) for tail harness application. Adequate depth of anesthesia was confirmed by the absence of the pedal withdrawal reflex. Animals were then suspended by the tail for 3 h once daily for 15 consecutive days, with hindlimbs elevated and without ground contact, while allowing forelimb locomotion for access to food and water. Control animals underwent identical daily handling and tail harness procedures but were not suspended. Both groups were fully awake during the unloading/handling period. The intermittent unloading protocol (3 h/day for 15 days) was specifically chosen to model early-stage disuse conditions rather than advanced atrophy. Continuous unloading models are known to induce more severe muscle loss but may also introduce systemic stress responses. Therefore, the present model was designed to reflect clinically relevant short-term immobilization scenarios frequently encountered in orthopaedic clinical practice, such as the early phase of fracture casting, protected weight-bearing after ligamentous repair, or early postoperative inactivity following major joint surgery.

Food and water intake were monitored daily, and no significant differences were observed between groups.

Blood Sampling and Serum Klotho Measurement

At the end of the 15-day intervention, blood samples were collected between 09:00 and 11:00 a.m. under deep anesthesia induced with ketamine (75–90 mg/kg) and xylazine (5–10 mg/kg, intraperitoneal). Euthanasia was performed under deep anesthesia by exsanguination via cardiac puncture, and death was confirmed by cessation of heartbeat and respiration. Samples were allowed to clot for 30 min at room temperature and then centrifuged at 3000 rpm for 15 min at 4 °C. Serum was aliquoted and stored at −80 °C until analysis.

Serum Klotho concentrations were measured using a commercially available sandwich ELISA kit specific for rat Klotho (Immuno-Biological Laboratories, Japan) according to the manufacturer's instructions. All samples were analyzed in duplicate, and investigators were blinded to group allocation.

Histopathological Analysis

Histological analyses were performed by investigators blinded to the experimental groups. EDL and soleus muscles were fixed in 10% buffered formalin for 48 h, embedded in paraffin, and sectioned transversely (5 µm). Sections were stained with hematoxylin–eosin (H&E) for morphological evaluation.

For morphometric analysis, six non-overlapping fields per muscle per animal were randomly selected at 40× magnification. Muscle fiber cross-sectional area (CSA) was measured using ImageJ software (version 1.54p, NIH, USA). Fiber borders were manually traced after calibration with a stage micrometer. Prespecified exclusion criteria included obliquely sectioned, longitudinally cut, necrotic, or split fibers, and areas containing artifacts.

In total, 1,922 EDL fibers and 1,834 soleus fibers were analyzed.

Masson's trichrome staining was performed on adjacent sections for qualitative assessment of interstitial collagen deposition.

To reduce measurement bias, morphometric analyses were performed by investigators blinded

to group allocation. All CSA measurements were conducted by the same observer using standardized ImageJ settings. To assess intraobserver reliability, ten randomly selected images were reanalyzed one week later.

Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics version 22.0 (IBM Corp., Armonk, NY, USA). Data distribution was evaluated using the Shapiro–Wilk test. Between-group comparisons were conducted using an independent samples t-test; Welch's t-test was applied when homogeneity of variances was not met. Data are presented as mean ± standard deviation (SD). All tests were two-tailed. Exact p-values were reported for all primary comparisons, and a p-value < 0.05 was considered statistically significant. Associations between serum Klotho levels and morphometric parameters were assessed using Pearson correlation analysis.

A priori sample size estimation was performed using G*Power software (version 3.1, Heinrich-Heine-Universität Düsseldorf, Germany). Based on an expected large effect size derived from previous experimental studies investigating skeletal muscle atrophy models, a minimum sample size of seven animals per group was estimated to achieve 80% statistical power with a two-sided significance level of 0.05. Therefore, eight animals were included in each group.

RESULTS

Quantitative morphometric analysis demonstrated a reduction in muscle fiber cross-sectional area (CSA) in both the extensor digitorum longus (EDL) and soleus muscles following intermittent hindlimb unloading. Compared with control animals, the disuse group exhibited a 2.74% decrease in mean CSA in EDL fibers and a 4.57% decrease in soleus fibers (Fig. 2A, B). Although the magnitude of reduction was modest, the differences were consistent and statistically significant ($p < 0.05$ for both comparisons; Fig. 2), suggesting early structural adaptation to disuse rather than advanced muscle degeneration.

Histological examination of hematoxylin–eosin (H&E)–stained sections from control animals revealed preserved muscle architecture, characterized by relatively uniform polygonal fibers with peripheral nuclei and no evident inflammatory infiltration (Fig. 4A, C). In contrast, muscle sections from HU rats showed mild structural alterations, including slightly reduced fiber caliber, focal architectural irregularity, and occasional mononuclear inflammatory cells in both EDL and soleus muscles (Fig. 4B, D).

Masson's trichrome staining revealed no apparent interstitial fibrosis in control muscles (Fig. 5A, C). In the disuse group, a mild increase in interstitial connective tissue was observed, particularly in the soleus muscle (Fig. 5B, D). As collagen deposition was assessed qualitatively, these findings are descriptive.

Scatter and distribution plots of individual CSA values demonstrated partial overlap between groups; however, the overall distribution shifted toward smaller fiber size in the disuse group (Fig. 2).

Serum Klotho concentrations were significantly lower in the disuse group compared with controls (control: 8.21 ± 0.81; disuse: 7.44 ± 0.54; $p = 0.045$, two-tailed) (Fig. 3A). Individual data points

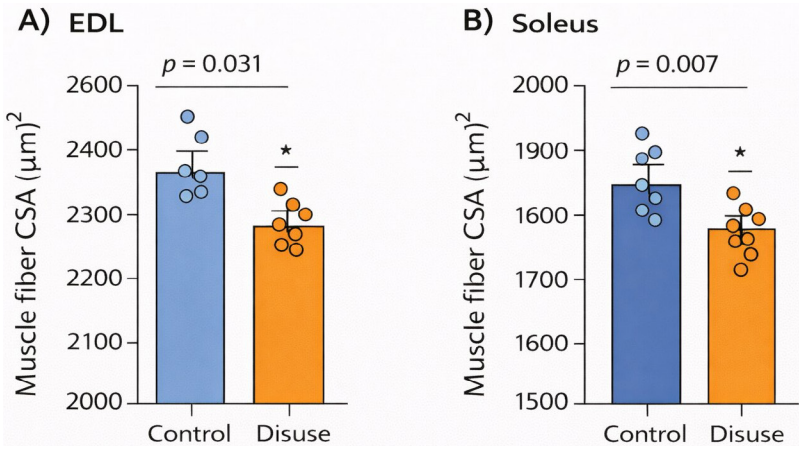

Fig. 2 — Quantitative analysis of muscle fiber cross-sectional area (CSA). (A) Extensor digitorum longus (EDL) muscle and (B) soleus muscle. Data are presented as mean ± standard deviation (SD) with individual data points (n = 8 per group). Between-group comparisons were performed using an independent samples t-test.

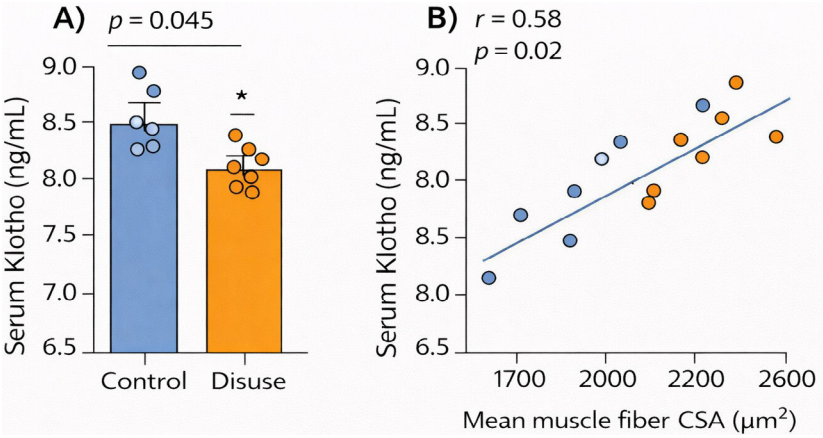

Fig. 3 — Serum Klotho concentrations and correlation analysis.
(A) Circulating Klotho levels in control and disuse groups. Data are presented as mean ± standard deviation (SD) with individual data points (n = 8 per group).
(B) Pearson correlation between serum Klotho concentrations and mean muscle fiber cross-sectional area (CSA) across all animals.

demonstrated a downward shift in circulating Klotho levels in suspended animals (Fig. 3A).

Pearson correlation analysis revealed a moderate positive association between serum Klotho concentrations and mean muscle fiber CSA (r = 0.58, p = 0.02; Fig. 3B).

DISCUSSION

Aging is associated with progressive decline in musculoskeletal function and increased vulnerability to orthopedic conditions such as fractures, reduced mobility, and postoperative complications. In this context, identification of circulating candidate biomarkers reflecting tissue-level changes has become increasingly important. Klotho has emerged as a longevity-associated protein, and circulating levels have been reported to decline with advancing age[27].

Although klotho is predominantly produced in the kidney, it has been detected in several extrarenal tissues, including skeletal muscle[28]. Experimental evidence indicates that klotho may contribute to muscle homeostasis by modulating oxidative stress, fibrosis-related pathways, and myogenic signaling[2,29]. Impaired klotho signaling has been associated with reduced muscle mass and compromised regenerative capacity in experimental models, whereas klotho overexpression or supplementation appears to enhance muscle regeneration under certain conditions[30,31].

In orthopaedic clinical practice, muscle atrophy is a common and debilitating consequence of immobilization, whether due to fracture casting, ligamentous injuries, or the protected weight-bearing periods following major joint surgery. Recent literature emphasizes that even short-term disuse can trigger significant molecular and structural shifts in skeletal muscle, complicating the postoperative rehabilitation trajectory. Integrating biological correlates, such as circulating Klotho, into the clinical assessment of disuse-related changes could potentially refine our understanding of muscle health during recovery[32,33].

Most previous studies have focused on the relationship between klotho and age-related muscle decline. However, it remains unclear whether circulating klotho responds to acute, non-age-related muscle stress such as disuse. The present study addressed this question using an intermittent hindlimb unloading model in young adult rats.

Our findings suggest that short-term intermittent unloading induced mild but significant reductions in muscle fiber cross-sectional area in both EDL and soleus muscles (Fig. 2), accompanied by early structural alterations on histological examination (Fig. 4 and Fig. 5). Importantly, these morphological changes were associated with a significant decrease in circulating Klotho levels (Fig. 3A). Furthermore, a moderate positive correlation was observed between serum Klotho concentration and muscle fiber size (Fig. 3B), suggesting an association between systemic Klotho availability and muscle structural integrity.

From a clinical perspective, these findings are particularly relevant in orthopedic settings, where short periods of immobilization are common following trauma or surgery. Even limited disuse may initiate early biological changes at the muscle level. However, the relatively small magnitude of structural alterations observed in this study suggests that these changes likely represent an early and potentially reversible phase rather than advanced muscle loss. Early identification of biological changes associated

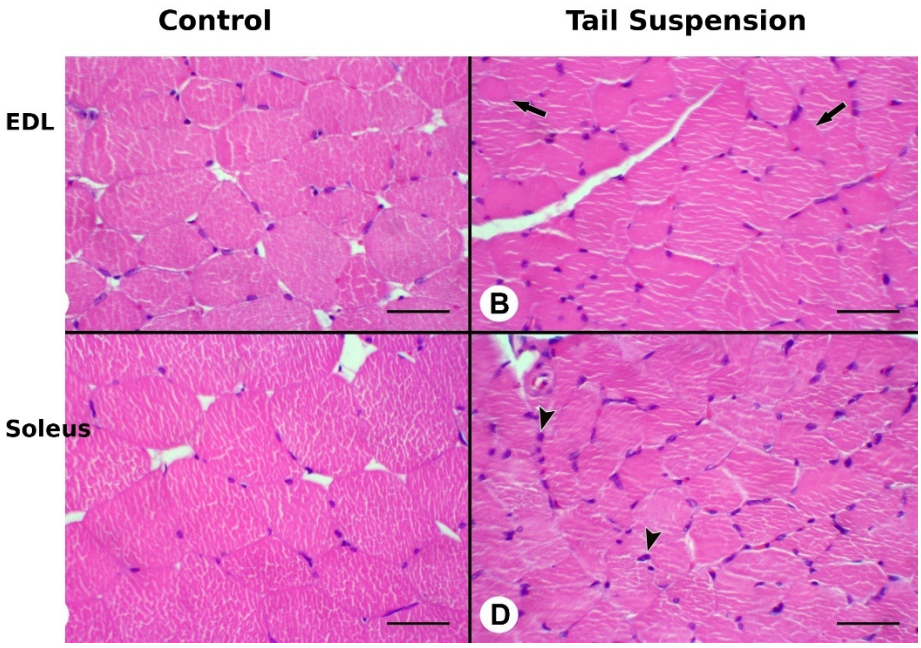

Fig. 4 — Representative hematoxylin–eosin (H&E)–stained sections of skeletal muscle (200×).
(A, C) Control EDL and soleus muscles.
(B, D) Disuse of the EDL and soleus muscles. Sections show muscle architecture and fiber morphology following intermittent unloading.

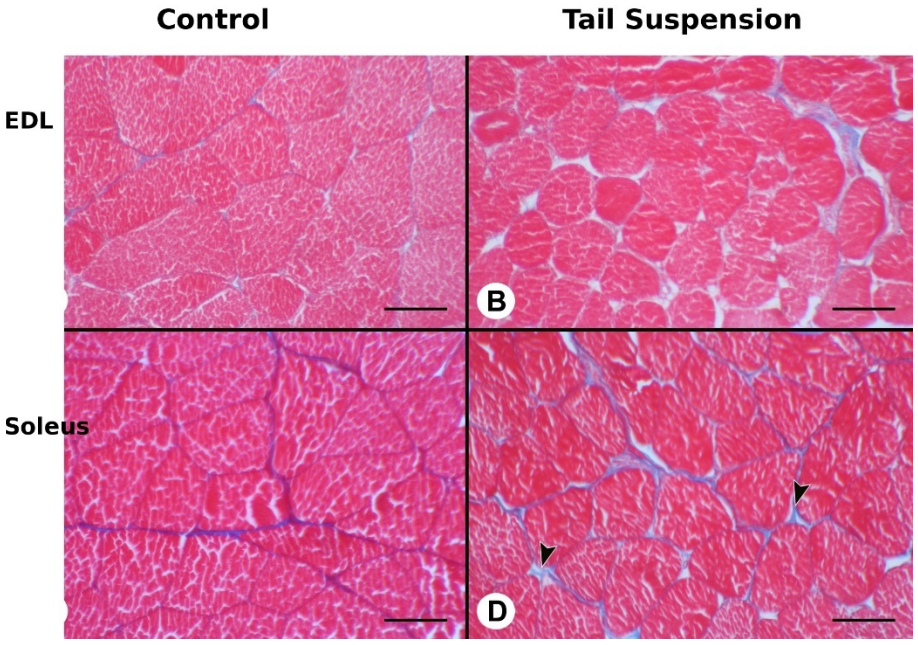

Fig. 5 — Representative Masson's trichrome–stained sections of skeletal muscle (200×).
(A, C) Control EDL and soleus muscles.
(B, D) Disuse of the EDL and soleus muscles. Sections illustrate interstitial connective tissue distribution following unloading.

with disuse may help guide rehabilitation strategies and optimize functional recovery, particularly in elderly orthopaedic patients.

The magnitude of CSA reduction was modest, which likely reflects the short-term and intermittent nature of the unloading protocol. Continuous hindlimb suspension models typically produce more pronounced atrophy. The intermittent unloading protocol used in this study was designed to reproduce early stages of disuse while minimizing systemic stress that may occur in continuous suspension models. Despite the relatively small structural changes, circulating Klotho levels showed a measurable decline, which may indicate that Klotho is sensitive to early disuse-induced

muscle remodeling. However, its role as a clinically applicable biomarker remains to be established.

The mild increase in interstitial connective tissue observed, particularly in the soleus muscle (Fig. 5), may represent early extracellular matrix remodeling. However, fibrosis was evaluated qualitatively, and quantitative collagen assessment was not performed.

Several limitations should be acknowledged. The sample size was limited, and causal relationships between Klotho reduction and muscle atrophy cannot be established. Wet muscle weight or whole-muscle mass was not measured; therefore, we cannot determine whether the modest CSA reductions translated into measurable loss of total muscle mass. Molecular markers of muscle proteolysis or regeneration were not assessed, and intramuscular Klotho expression was not evaluated. Therefore, the underlying mechanisms linking unloading and reduced circulating Klotho remain speculative.

These findings should be interpreted with caution, as the observed associations do not imply causality but rather suggest a potential link between systemic Klotho levels and early muscle structural changes.

CONCLUSION

Partial hindlimb unloading induced early disuse-related muscle atrophy in both fast- and slow-twitch muscles and was associated with a significant reduction in circulating Klotho levels. Klotho may represent a candidate biomarker of early muscle disuse; however, further mechanistic and clinical studies are required before its diagnostic or therapeutic relevance can be established.

Funding: This study was supported by the Fırat University Scientific Research Projects Unit.

Ethics approval and consent to participate: All animal procedures were carried out in accordance with relevant guidelines and regulations for the care and use of laboratory animals. The study protocol was approved by the Laboratory Animal Care and Use Committee of Fırat University Faculty of Medicine (approval date: 17 July 2023; approval number: 17217). All methods are reported in accordance with the ARRIVE guidelines (https://arriveguidelines.org).

No ether was used for anesthesia or euthanasia at any stage of the study. Anesthesia was achieved using ketamine (75–90 mg/kg) and xylazine (5–10 mg/kg) administered intraperitoneally, and euthanasia was performed under deep anesthesia by exsanguination via cardiac puncture.

Funding: This study was supported by the Fırat University Scientific Research Projects Unit.

Acknowledgements: The authors gratefully acknowledge the support of the Fırat University Scientific Research Projects Unit.

Competing interests: The authors declare that they have no competing interests. All authors read and approved the final manuscript.

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