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VOLUMETRIC MUSCLE LOSS IN RATS: A CLINICAL AND PARACLINICAL STUDY

PIERDEREA MUSCULARĂ VOLUMETRICĂ LA ȘOBOLANI: UN STUDIU CLINIC ȘI PARACLINIC

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ABSTRACT | REZUMAT

Volumetric muscle loss (VML) represents a significant loss of skeletal muscle mass, leading to severe functional impairment. This condition poses a significant challenge in both human and veterinary medicine. Working dogs, integral to various sectors, are particularly susceptible to VML due to their demanding roles. Skeletal muscle has an intrinsic regenerative ability, yet current treatments for VML fall short of achieving complete functional recovery. Tissue engineering and regenerative medicine offer promising avenues for addressing this critical issue. The present study investigates various rodent tibialis anterior (TA) muscle injury models, with particular emphasis on one specific technique. Two biomimetic sponges were used to treat VML defects in TA muscle in male Wistar rats. Locomotor function was assessed clinically, and TA muscle morphology was monitored using ultrasound at 4, 6, 8, and 12 weeks post-injury. The contra-lateral TA muscle served as the untreated VML control. Compared to control groups, scaffold treatment markedly enhanced muscle regeneration in male rats with VML, evident in increased muscle mass and improved function from one-week post-implantation onwards. In conclusion, the model's straightforward design, consistent outcomes, and clinical applicability make it a prime choice for a standardised VML research model.

Keywords: volumetric muscle loss, tissue engineering, skeletal muscle regeneration, biomaterials

Pierderea volumetrică a mușchilor (VML) reprezintă o pierdere semnificativă de masă musculară scheletică, care duce la o afectare severă a funcției musculare. Această problemă constituie o provocare majoră atât în medicina umană, cât și în cea veterinară. Câinii de lucru, esențiali în diverse sectoare, sunt deosebit de vulnerabili la VML din cauza activităților solicitante. Deși mușchiul scheletic are capacitate regenerativă intrinsecă, tratamentele actuale pentru VML nu asigură o recuperare funcțională completă. Ingineria tisulară și medicina regenerativă oferă soluții promițătoare. Studiul de față analizează mai multe modele experimentale de leziuni musculare la nivelul mușchiului tibial anterior (TA) la șobolani, punând accent pe o tehnică specifică. Doi bureți biomimetici au fost utilizați pentru a trata defectele de VML din mușchiul TA la șobolanii Wistar masculi. Funcția locomotorie a fost evaluată clinic, iar morfologia musculară a fost monitorizată ecografic la 0, 4, 6, 8 și 12 săptămâni post-leziune. Mușchiul TA contralateral a servit drept control pentru VML netratat. Comparativ cu grupurile de control, tratamentul cu biomateriale a îmbunătățit semnificativ regenerarea musculară la șobolanii masculi cu VML, evidențiindu-se prin creșterea masei musculare și îmbunătățirea funcției de la o săptămână post-implantare. În concluzie, designul simplu al modelului, rezultatele consecvente și aplicabilitatea clinică îl recomandă ca model standardizat pentru cercetarea VML.

Cuvinte cheie: pierdere volumetrică a mușchilor, inginerie tisulară, regenerare musculară scheletică, biomateriale

Dogs serving in both military and civilian capacities, including protection, detection, search and rescue, or service work, face elevated injury risks while carrying out their duties (e.g., disability or even death) (1). The demanding nature of their work puts them at a higher risk of harm from hazardous conditions and toxic agents. Gunshot wounds, especially from high-powered projectiles, can lead to bone fractures or soft tissue destruction, causing significant damage. Bite injuries comprise 10-15% of traumatic cases in cats and dogs (2,3). Capable of exerting a force of 150-450 pounds per square inch, canine teeth can cause severe tissue trauma through a combination of crushing, tearing, and puncture injuries (4,5). Significant muscle loss can occur both as a consequence of orthopaedic surgery and through the removal of large tumours (6). All of these lesions can result in skeletal muscle loss, a condition termed volumetric muscle loss (VML), which occurs when skeletal muscle is lost, leading to functional impairment (7-10).

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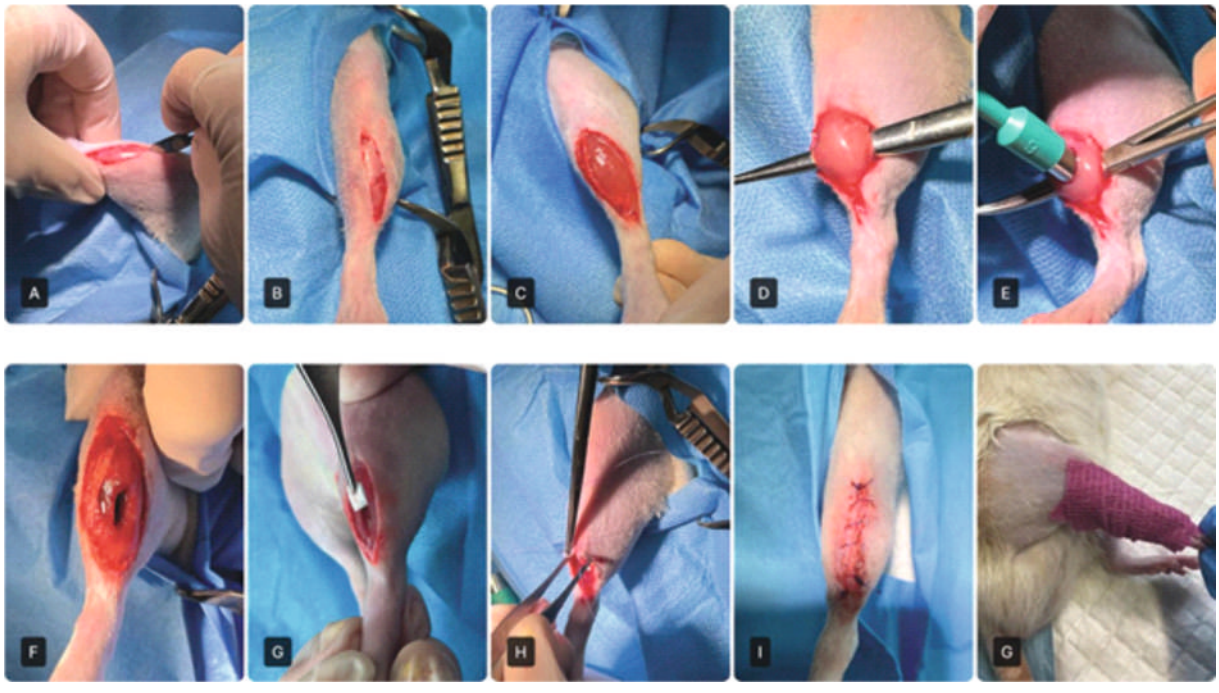

Fig. 1. Representation of the rat TA muscle surgical procedure.
Around 20% of the TA muscle was removed to induce a non-recoverable VML injury

Skeletal muscle possesses a remarkable ability to regenerate after minor injuries. Given its high metabolic demands, skeletal muscle relies on a dense capillary network for nutrient and oxygen supply to sustain function. In response to these injuries, damaged fibres degenerate, triggering an inflammatory response that recruits immune cells to clear debris and promote repair. Simultaneously, satellite cells, the muscle's stem cells, are activated. These cells proliferate, differentiate into myoblasts (immature muscle cells), and fuse to form new muscle fibres. As the repair progresses, the new fibres align, mature, and integrate with surrounding tissue, while blood vessels and nerves regrow to restore function (11). Proper healing is essential for restoring and maintaining long-term muscle function after injury. The scar tissue that forms typically lacks adequate blood vessel formation and shows significant deficiencies in tissue regeneration (12). However, in cases of substantial muscle loss, the body's regenerative capacity is overwhelmed, leading to an abnormal inflammatory response and excessive collagen deposition. This results in a fibrotic healing process and subsequent chronic muscle weakness (13-15).

The architecture of skeletal muscle can experience substantial changes, especially following muscle reconstruction through tissue engineering approaches, such as VML injury (16). In these models, different types of scaffolds, sometimes combined with cells, are implanted into the injured area. Along with the regenerative potential of the host muscle, these materials may or may not support the regeneration of new muscle tissue (17). In these cases, it is essential not only to evaluate functional recovery but also to assess whether the regenerated tissue resembles the structure of healthy muscle. In both a human trial and an equine model, ultrasound was used to assess the integration of scaffolds into skeletal muscle, primarily to identify the precise location of the implants (18, 19). Ultrasound (US) serves as a crucial tool for evaluating both normal and abnormal skeletal muscle structure. US examinations primarily focus on diagnosing and monitoring neuromuscular disorders and traumatic injuries (20-22).

This study aimed to evaluate the effectiveness of a VML protocol and the techniques used to assess it, with the ultimate goal of testing novel biomaterial engineering strategies in future research. Importantly, the protocol was designed to minimise complications and ensure animal welfare.

MATERIALS AND METHODS

Animal Care

The method employed in this study follows the approach outlined by Xiaowu Wu et al. (2012) (23) because it is a reliable and technically simple method for inducing VML injury (23). This work was conducted in compliance with the European guidelines and rules 337, as established by the EU Directive 2010/63/EU and the Romanian law 43/2014. All procedures were approved by the Research Ethics Committee of the University of Agricultural Sciences and Veterinary Medicine (USAMV) Cluj-Napoca, Romania, and they were authorised by the State Veterinary Authority (ethical approval number 369 /13.06.2023). Adult male Wistar rats were purchased from Cantacuzino Institute (Bucharest, Roma-nia), and housed (12-hour light–dark cycle) in the Establishment for Breeding and Use of Laboratory Animals of USAMV. All the animals were fed with sterile, acidified water (pH

2.7–3) and sterile, standard rodent chow, ad libidum. Animals were randomly assigned to three experimental groups: untreated VML control, and two sponge-treated VML.

Anaesthesia and Analgesia

A total of 20 male rats, weighing 360 ± 50 g, underwent surgery under continuous inhalation of isoflurane (1.5-2.5%). An ophthalmic ointment was topically applied to both eyes of the anesthetized animals. Anaesthesia depth was assessed by toe pinch response, and core temperature was maintained with a heated water perfusion system. Preoperative analgesia was provided through a subcutaneous injection of slow-release buprenorphine (0.1 mg/kg), followed by a quick-release buprenorphine (0.1 mg/kg) at 12- and 24-hours post-surgery. A qualified veterinary staff monitored animals daily for pain and distress, determining the need for further analgesia. No animals required additional pain management beyond the initial 24-hour period.

Tibialis anterior VML Surgery

The surgical site was aseptically prepared by depilation and application of an antiseptic solution (Lifo-Scrub). Subsequently, the area was disinfected with 70% alcohol using a circular motion, progressing outward from the incision.

The surgery area was isolated with a sterile drape. A longitudinal skin incision was made just below the knee joint, on the lateral side, to expose the tibialis anterior (TA) muscle (Figs 1A, B). This was followed by blunt separation of the fascia lata covering the TA muscle (Fig. 1C). To create a stable surface for the surgical defect, the TA muscle was elevated with a flat instrument, carefully avoiding the underlying extensor digitorum longus (EDL) muscle (Fig. 1D). A full-thickness defect from the belly of the TA was excised using a 6-mm biopsy punch (Kai Medical), removing approximately ~20% of the muscle mass (Fig. 1E). For the sponge treatment group, the biomaterials were implanted at the injury site, while for the no treatment group, the injury received no intervention (Fig. 1F, G). The fascia was closed with 6-0 Vicryl sutures, followed by skin closure with 6-0 Prolene sutures (Figs 1H, I). A compression bandage was then wrapped around the lower leg for 5 min (Fig. 1G). The animals were allowed to recover for 4, 6, 8, and 12 weeks and euthanised via exsanguination followed by cervical dislocation. The wound showed no signs of redness or infection, and no obvious systemic reactions were found. To ensure treatment group consistency, both hind limbs underwent surgery. Bilateral surgery aligns with ethical considerations by minimising animal use (24).

General Observation

The locomotor function examination was conducted from a distance, focusing on assessing the various postures adopted by the rats (25). In the quadrupedal position (1), the examination helps identify any reluctance or difficulty in bearing weight on a limb, often indicated by a limb positioned laterally from the body. Observing the rat's movements (2) allows for the detection of lameness through dynamic assessment, particularly evidenced by erratic movements and lumbar kyphosis. Finally, the standing position (3) indicates an almost complete absence of pain, as the rat can support twice as much weight on its hind limbs.

Ultrasound in Situ

B-mode US were performed on weeks 0, 4, 6, 8, and 12. After anaesthetic administration, the animals were positioned supine, the area for ultrasound examination was shaved, and Aquasonic® 100 ultrasound transmission gel was applied before initiating the ultrasound evaluation. The same operator (specialist radiologist) conducted all ultrasound analyses to ensure consistency.

For this analysis, a General Electric ACUSON Juniper ultrasound machine (Siemens) equipped with a high-frequency linear probe (8–18 MHz) was used. Bilateral images of the anterior region of each subject's hind limb were captured in both transverse and longitudinal planes in B-mode. In B-mode, the region was evaluated with the following settings: tissue harmonic imaging (THI) frequency of 16 MHz, gain set to 32, enhancement at 3, an average of 2, and a dynamic range of 96 dB. US images were evaluated based on a classification system to assess levels of increased echogenicity, echotexture distortion, and vascularity, categorised as follows: Grade 0 (normal), Grade 1 (mild), Grade 2 (moderate), and Grade 3 (severe/high). The scanning area included both the biomaterial implant site and the adjacent host tissue, allowing for a comprehensive assessment of tissue integration and interaction between the implanted material and surrounding structures.

Tibialis anterior muscle analysis

The rats were euthanised at 4 (n = 5), 6 (n = 5), 8 (n = 5) and 12 weeks (n = 5) after surgery. For all dissections, hair removal from the hindlimb was required. This was achieved by shaving the area with a scalpel. All of the manipulations were performed without touching the muscle, to prevent any damage that would compromise myofiber integrity. A longitudinal incision was made along the lateral side of the left and right hind limbs, extending from the knee to the ankle. The skin was separated from the underlying fascia through blunt dissection. The lower tendon was cut at the most distal part from the muscle. The muscle was carefully lifted away from the leg while simultaneously releasing the connective tissue on both sides of the TA. Since the upper tendon of the TA was not always visible in all rats, fine-tip scissors were used at the knee level to cut the TA muscle as close to the base as possible. The TA muscle was positioned on a longitudinally split cork and secured with two pins, placed at the top and bottom.

RESULTS AND DISCUSSION

Locomotory Function

Throughout all observation periods, the walking and standing behaviours of the rats were monitored, with images presented in Figure 2 for reference. Rats in each

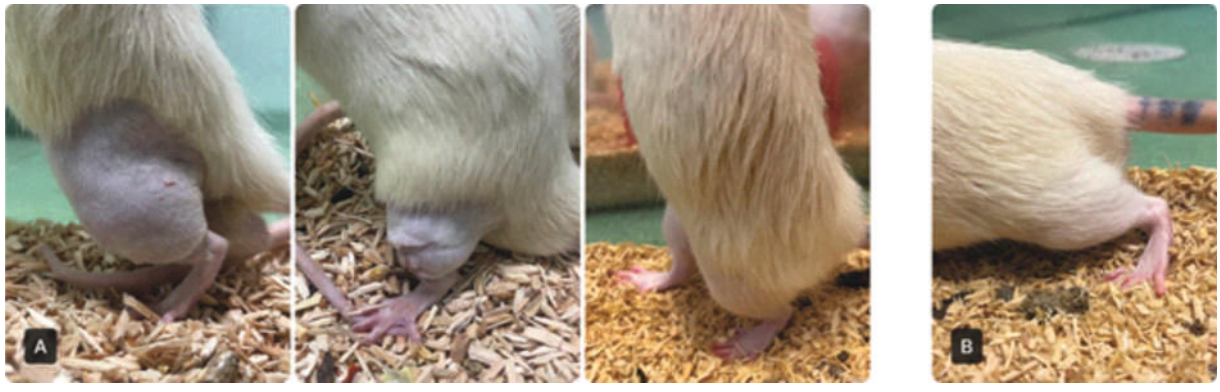

Fig. 2. General observation of the movement of rats at each of the designated intervals after surgery. Images of standing (A) and walking (B) rats

group maintained balanced standing postures, suggesting effective recovery of the injured TA muscle's innervation function. All rats demonstrated normal muscle function post-surgery, showing no signs of lameness. They were able to bear weight on their paws, walk without limping, and stand upright as early as day zero, with no indications of pain. For those with VML only, no loss of muscle function was observed, only mild discomfort that persisted for a few days after the intervention. In addition, there were no obvious abnormalities in their behaviour or diet. This outcome is promising, as it supports the formation of a muscle defect that can regenerate without causing pain or irreversible damage to the opposing limb. The success is attributed to a precise and clean muscle excision at an optimal volume. Additionally, the lack of a foreign-body inflammatory response suggests that the biomaterials were well-integrated in vivo. These results imply that both biomaterials used did not cause pain or compromise muscle function.

Gross morphology of VML injured tibialis anterior muscle

At the implantation site, changes in sample size and surrounding inflammation were monitored at 4, 6, 8, and 12 week time points. The TA muscle experienced significant structural deformation and gross morphological alterations as a result of VML injury (Fig. 3).

In muscles with untreated VML injury, a longitudinal tear was frequently observed where the defect initially occurred (Fig.s 3I, II, III - arrow). A more defined scar formation was observed, accompanied by a reduction in tissue loss at the site of the injury. The fascia became more fibrous and adhered to the underlying tissues, which may, in time, limit the flexibility and function of the TA muscle. This fibrotic tissue could potentially lead to long-term discomfort or mobility issues. At 4 and 6 weeks post-injury, pronounced adhesions were noted, whereas at 8 and 12 weeks, the fibrosis was less severe. The presence of these adhesions suggests a persistent functional deficit. Both biomaterial-treated groups, as identified by their functional responses, seemed to partially address this gap, reducing the atrophic appearance of the muscle. In line with this, the biomimetic sponges in group A exhibited excellent integration with the surrounding tissue, and the scarring appeared more uniform, potentially reducing the risk of adhesions. At the 4- and 6-week post-injury time points, the onset of vascularisation and improved alignment of fibres were clear indicators of active muscle regeneration (Fig. 3II). In contrast, group B showed incomplete biomaterial integration, as the outline of the material remained visible, suggesting less efficient incorporation into the surrounding tissue (Fig. 3III). The sponges were visible upon TA exposure and appeared intact (Figs 3II, III).

At 8 and 12 weeks, the biomaterials are absorbed and replaced by tissue resembling native muscle. At 8 weeks, the biomaterial in group A is nearly fully replaced by regenerated tissue, with muscle fibres aligning more closely to their native structure. By 12 weeks, integration improves further, enhancing the tissue's stability and elasticity. In contrast, group B shows incomplete integration at 8 weeks, with visible biomaterial remnants and less organised tissue. By 12 weeks, while integration improves, scarring and functional recovery remain less optimal compared to group A.

Skeletal Muscle Imaging

The main objective of the study is to assess structural changes in the muscle, such as tissue degeneration, regeneration, swelling, or replacement with fibrous or fatty tissue, all of which are reflected in altered ultrasound patterns. The sponges were observed as multiple stacked linear and hyperechoic structures close to the surface of the muscle and were visible up to 4 weeks post-repair during longitudinal imaging.

The two biomaterial groups showed distinct results in tissue regeneration. The group treated with biomaterial A demonstrated near-complete tissue regeneration, while the group treated with biomaterial B exhibited some degree of regeneration but also signs of incomplete tissue recovery. In contrast, the untreated VML group continued to develop fibrosis. These variations were clearly reflected in the ultrasound findings, which indicated differing tissue responses to the two biomaterials compared to the untreated VML group.

- **At 4 weeks**

Group A showed faster and more pronounced regeneration. At 4 weeks, the injury margins were well defined, and the echostructure was more organised, with clearer muscle fibre definition and perimysial pa-

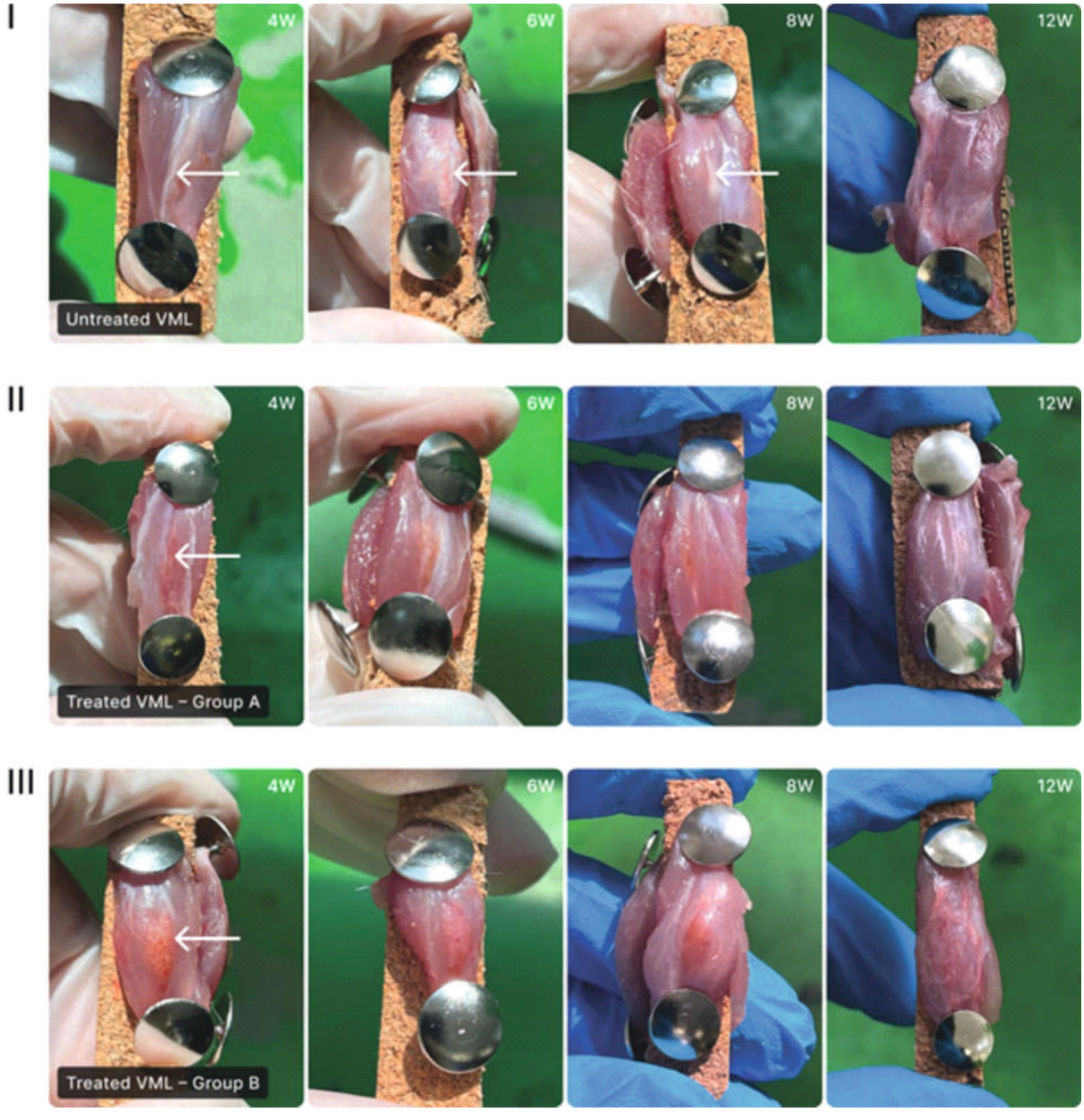

Fig. 3. TA muscle analysis at 4, 6, 8 and 12 weeks.
Images of TA muscle between (I) untreated VML and (II & III) operative sides

tterns. In Group B, regeneration signs appeared by week 4, with decreased lesion size and increased echogenicity, but the echostructure remained disorganised, indicating ongoing remodelling. The implantation area was hyperechogenic. Both sample groups exhibited isoechoic signals within the muscle layer, suggesting implant presence, with group B showing more pronounced visibility. (Figs 4A, B - arrow). The untreated VML group exhibited persistent damage and inflammation, characterised by large, disorganised lesions, disrupted perimysial architecture, and a poorly defined epimysium. These structural irregularities contributed to significant fibrosis in the affected muscle tissue.

- **At 6 weeks**

By 6 weeks, Group A showed near-complete lesion closure and a highly organised echostructure, with muscle fibre and perimysial patterns closely resembling healthy muscle tissue. Group B exhibited continued improvement, with a more organized echostructure and clearer muscle fibre definition. While perimysial patterns were still less distinct than in Group A, they were more recognisable. In contrast, the untreated VML group showed disorganised echostructure, indicating ongoing inflammation and fibrosis, with continued perimysial disruption and indistinct epimysium. The injury area exhibited variable morphology, increased echogenicity, and severely distorted echotexture.

- **At 8 weeks**

Group A achieved full recovery, with complete lesion closure, well-aligned muscle fibres, and minimal fibrosis,

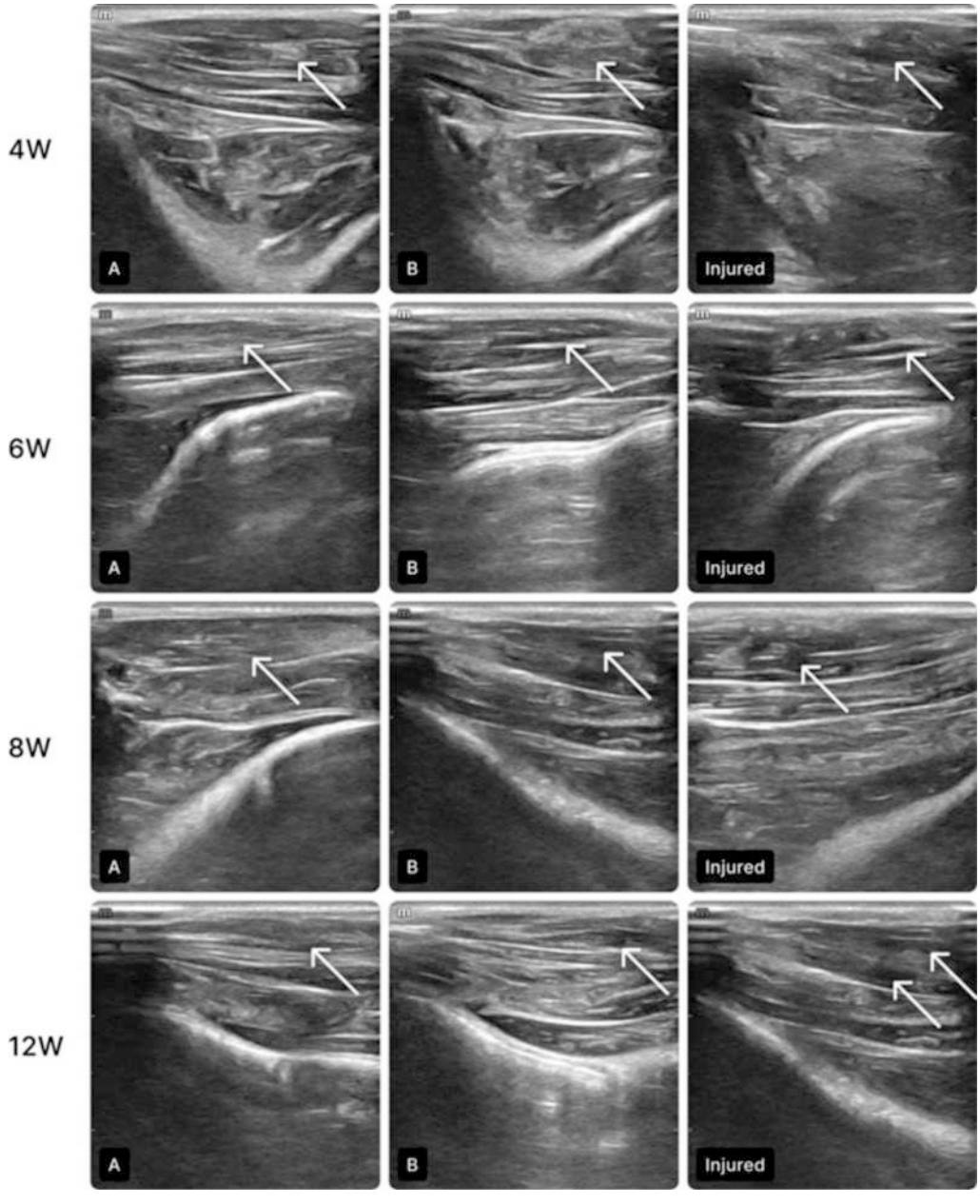

Fig. 4. Examination of B-mode US of two types of scaffolds and untreated VML at 4, 6, 8, and 12 weeks

closely resembling healthy muscle tissue. Group B demonstrated considerable progress, with reduced lesion size and better-organised fibres. However, mild fibrosis and inflammation persisted. The untreated VML group still exhibited large, disorganised lesions with ongoing inflammation and fibrosis, reflecting limited healing.

- **At 12 weeks**

Group A remained fully recovered, with no visible lesions and highly organised muscle fibres, indicating complete regeneration. Group B exhibited near-total recovery, with closed lesions, more organised muscle fibres, and reduced fibrosis, although some minor inflammation remained. The untreated VML group showed some recovery, with a reduction in lesion size and improved tissue organisation compared to earlier time points. The small gaps observed in the untreated VML group indicate ongoing inflammation and fibrosis, high-lighting incomplete tissue repair and slow healing processes.

Discussion

The current literature on the in vivo degradation of sponge biomaterials reveals a lack of standardised methodologies and consistent experimental evaluation techniques. Non-invasive imaging techniques are essential for conducting objective, longitudinal analyses of muscle volume and quality, offering valuable insights into the effectiveness of new treatments for muscle injuries (26). In preclinical VML studies, computed tomography (CT) is the most commonly used imaging tool for tracking muscle volume (27). However, in clinical se-

ttings, particularly for VML trauma, the need for retrospective analysis and patient transfer to CT imaging limits the ability to carry out accurate longitudinal studies (26). Ultrasound, on the other hand, offers a rapid, non-invasive, and cost-effective alternative for assessing muscle health in VML injuries. While US has been employed in clinical VML studies, it has not yet been widely used in rodent preclinical models (28). This technique enables both quantitative measurements, such as muscle cross-sectional area to assess muscle maintenance or recovery, and qualitative assessments, such as evaluating echogenicity to determine muscle structure and integrity (29). In this study, we correlated B-mode US findings with clinical observations and post-mortem changes at the defect site in the anterior tibial muscles of rats at 4, 6, 8, and 12 weeks following the implantation of various scaffolds into a VML-induced muscle defect. US analysis demonstrated that muscles treated with biomaterials showed substantial recovery, characterised by reduced lesion size and well-organised tissue that closely resembled healthy muscle. In contrast, the untreated VML group exhibited a disorganised echostructure, larger lesions, and persistent signs of fibrosis and inflammation. VML involves the intentional removal of muscle tissue, accomplished through various tools and methodologies. For instance, the excision of 40% of the TA muscle has been shown to reduce functional strength by approximately 40% compared to controls, while a related study reported a 30% reduction in strength after removing 30–50% of the same muscle (30, 31). These findings underscore that the relationship between muscle removal and functional strength loss is not consistently linear, as outcomes are significantly influenced by variables such as the geometry of the injury and the anatomical characteristics of the affected muscle (32).

To induce VML, studies employ diverse methods ranging from excising small portions, like 20% of the TA muscle, to removing up to 75% of the quadriceps compartment. Techniques vary, including using defined volume measurements, such as excising 4 × 2 × 2 mm^3 of tissue with iris scissors or creating 2 mm punch biopsies (28, 33).

Alternatively, muscle mass-based methods are used, such as removing 20% of the middle third of the TA, with tissue mass estimated relative to the animal's body weight (34). While variations in injury methodology do not seem to cause significant changes in results, the approach that involves removing measured tissue volumes (length × width × height) is often favoured for its ability to ensure consistency and standardisation in experimental studies.

While muscles like the abdominal wall, latissimus dorsi, and quadriceps femoris have been used in VML research, the TA muscle is particularly advantageous due to its accessible surgical location and the measurable functional deficits it produces (35-37). Tissue engineering strategies for VML repair are commonly evaluated in small animal models (38).

The 6 mm punch biopsy technique was selected as it offered several distinct advantages: 1) By creating a centrally located muscle defect surrounded by intact tissue, it provided an optimal environment for studying regeneration. 2) Observations during the study suggested that the 20% defect was quickly filled with tissue, indicative of a natural healing response, potentially involving inflammation. The healing process for muscle injuries remains constant, regardless of their underlying cause (39, 40). Strains, which are particularly prone to recurring, require careful assessment of muscle strength before returning to full activity, according to some studies, to avoid re-injury (41,42).

Skeletal muscle fibrosis is recognised for hindering the healing and regeneration of muscle tissue, disrupting the muscle's microenvironment, and leading to the degradation of its architecture (43). The extensive fibrotic response that occurs after injury, without intervention, may not only limit rehabilitation efforts but also continue to negatively affect the remaining muscle, as it follows the natural progression of the injury. This ongoing process may prevent full recovery and functional restoration of the affected muscle. Clinical assessments of locomotor function revealed that the rats maintained normal movement without any visible signs of pain, confirming the human nature of the approach. Another significant strength of the protocol was its reliability and reproducibility; the punch biopsy consistently created uniform defects in the TA muscle across all subjects, ensuring consistency in the experimental model.

CONCLUSIONS

Overall, the results show that the scaffold material integrates effectively with the surrounding muscle tissue at the VML injury site, potentially facilitating tissue remodelling. This treatment helps reduce the severity of the injury, leading to improvements in muscle structure and function.

Despite its widespread clinical use, the application of ultrasound to biomimetic sponges has not been extensively studied. This research applied B-mode ultrasound to observe and semi-quantify the breakdown of scaffolds, comparing the findings with clinical evaluations and post-mortem assessments. The ultrasound successfully visualised the implanted scaffolds and allowed real-time tracking of their degradation in vivo. Aside from its utility in diagnosing neuromuscular conditions and monitoring muscle injuries, we consider diagnostic sonography to be an invaluable method for assessing the structure of muscle tissue through engineered techniques. There is a lack of articles addressing the use of ultrasound to monitor the disintegration of sponge materials. For this reason, the findings of this study could serve as a foundation for future research in this area.

A potential limitation of this study is the absence of a correlation between the histological data and the ultrasound results. More research is needed to establish how muscle ultrasound can impact clinical decision-making, improve patient outcomes, and offer cost-effective benefits, whether used independently or as part of a stepwise diagnostic process, with ultrasound as the initial, patient-friendly test.

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