Research Article

Development and Validation of a Multiparametric Semiquantitative Scoring System for the Histopathological Assessment of Ischaemia Severity in Skeletal Muscle

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Skeletal muscle is one of the most abundant and dynamic tissues of the body, with a strong regenerative capacity. Muscle injuries can occur as a result of a variety of events, including tissue ischaemia. Lower limb ischaemia occurs when there is an insufficient nutrient and oxygen supply, often caused by stenosis of the arteries due to atherosclerosis. The aim of this study was to develop and validate a multiparametric scoring tool for assessing ischaemia severity in skeletal muscle in a commonly used preclinical animal model. Tissue ischaemia was surgically induced in mice by ligation and excision of the femoral artery. Calf muscles were carefully dissected, prepared for histological analysis, and scored for inflammation, fibrosis, necrosis, adipocyte infiltration, and muscle fibre degeneration/regeneration. Kendall’s coefficient of concordance ($W$) showed a very good agreement between the appraisers when scoring each individual histological feature: inflammation ($W = 0.92, p \leq 0.001$), fibrosis ($W = 0.94, p \leq 0.001$), necrosis ($W = 0.77, p \leq 0.001$), adipocyte infiltration ($W = 0.91, p \leq 0.001$), and fibre degeneration/regeneration ($W = 0.86, p \leq 0.001$). Intrarater agreement was also excellent ($W = 0.94$ or more, $p \leq 0.001$). There was a statistically significant negative association between the level of muscle ischaemia damage and the calf muscle weight and skeletal muscle fibre diameter. Here, we have developed and validated a new multiparametric, semiquantitative scoring system for assessing skeletal muscle damage due to ischaemia, with excellent inter- and intrarater reproducibility. This scoring system can be used for assessing treatment efficacy in preclinical models of hind limb ischaemia.

1. Background

Skeletal muscle is the most abundant tissue in the body, comprising approximately 40% of the total body weight. Structurally, skeletal muscle is a highly organised tissue, which comprises several bundles of muscle fibers surrounded by connective tissue with different names (epimysium, perimysium, and endomysium) depending on the location [1] (Figure 1). Within the skeletal muscle, there is an abundant supply of blood vessels and nerves, which are essential for maintaining the principal muscle functions such as contraction, oxygen delivery, and waste removal [2]. Skeletal muscle is also a very dynamic tissue with a strong regenerative capacity in response to injury or disease. Regeneration of fibers mainly relies on resident muscle stem cells (MuSCs), also called satellite cells (SCs), which are localised between the basal lamina and the muscle fiber membrane [3]. SCs typically exist in a quiescent state but following injury SCs become activated, proliferate and give rise to myogenic precursor cells, known as myoblasts,

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repair phase, characterised by the activation, proliferation, and differentiation of SCs into myoblasts and then myotubes to replace damaged myofibers. The final phase involves extracellular matrix remodeling and maturation of regenerated myofibers with the recovery of muscle function. In most cases, this system leads to successful muscle repair and regeneration of the injured tissue. For instance, in minor or acute muscle injuries, the activation of the muscle repair and regeneration program often leads to full functional recovery. However, if the duration, frequency, and magnitude of the injury are too great, successful regeneration may not be achieved and instead injured tissue is replaced by connective tissue and fat [7].

Over the past years there has been a growing interest in understanding the cellular and molecular mechanisms underlaying skeletal muscle regeneration with the aim of developing novel therapies. In vivo preclinical animal models remain the optimal tools for assessing therapeutic efficacy of novel therapeutic products, which often requires a complete morphologic assessment of tissues for treatment group comparisons. Histopathological scoring is a tool by which semiquantitative data can be obtained from tissues [8]. It usually involves scoring of a lesion’s magnitude on an ordinal scale. Several multiparametric, semiquantitative standard scoring systems have been introduced previously for histopathological assessment of tissue lesions in different mouse models of disease [9]. However, fewer semiquantitative scoring systems have been described in the literature for assessing skeletal muscle damage [10–14].

Here, we have developed and validated a novel multi-parametric, semiquantitative scoring system which assesses histopathological parameters known to be present in ischaemic muscle tissue [15–17], with excellent inter- and intrarater reliability. This scoring system can be used for assessing the degree of muscle ischaemic damage and for the treatment group comparison in preclinical efficacy studies that use the murine model of hind limb ischaemia (HLI). The use of a reliable and standardised scoring system for a specific disease model will allow more meaningful comparison of results from different studies and laboratories.

2. Materials and Methods

2.1. Animals. Male 8–10 weeks old BALB/c nude mice were purchased from Envigo (United Kingdom) and were housed in a licensed preclinical facility at Biomedical Science, at the University of Galway, with monitoring and support from qualified animal technicians and a veterinary surgeon. Ethical approval was granted by the Institutional Animal Care Research Ethics Committee. Project authorisation was granted by the Health Products Regulatory Authority in Ireland.

2.2. Induction of HLI. Unilateral HLI was surgically induced in 8–10 weeks old male BALB/c nude mice. Animals were anaesthetised with 75 mg/kg Ketamine and 0.5 mg/kg Dormitor 10 solution injected subcutaneously. The femoral triangle in the left leg was exposed through an incision in the
2.6. Scoring System. A total of 70 skeletal muscle samples were scored. These comprised a selection of samples from nonischaemic skeletal muscles (normal) as well as ischaemic skeletal muscles with a wide range of ischaemic damage and regeneration to capture all the proposed levels (as shown in Table 1). Each muscle tissue sample was carefully observed under an Olympus Bx43 bright field microscope by three independent operators. Scoring of each sample was performed by visualising the entire calf muscle cross-sectional area directly under the microscope, not by scoring multiple single frame images taken from the same muscle section (i.e., fields of view). The researchers observed four separated cross-sections for each calf muscle, and a score was given that best represented that particular calf muscle. While in the majority of cases, the same degree of lesion was present across the four cross-sectional areas observed, it was possible that in some cases, the same degree of lesion was not present in all the cross-sections. In this scenario, the major score for that particular lesion was given as demonstrating the highest severity of the pathology present in the muscle. All the microscope magnifications can be used in order to obtain a general view as well as more detailed view of the regions of interest. However, a recommended magnification for scoring each parameter is detailed in Figure 3.

Figure 3 describes the proposed system of evaluation. Here, a detailed description and sample pictures of muscle
INFLAMMATION: The terminology ‘inflammation’ refers when leukocyte accumulations are part of an active inflammatory process in combination of other concurrent features such as vascular changes, presence of necrosis, fibrosis and disruption of muscle fibre architecture. Leukocyte ‘infiltration’ is most appropriately used when leukocyte accumulations are present in tissue without other disruption or pathology. Recommended microscope magnification for scoring is 10X.

0 Normal: Absence or minor presence of vascular associated leukocyte accumulations.
1 Mild: Localised or mildly scattered leukocyte accumulation in tissue, with no evidence of significant damage of muscle fibre bundles (e.g. leukocyte infiltration).
2 Moderate: Moderately scattered or multifocal cluster leukocyte accumulation, with disruption of several muscle fibres and with/without presence of active phagocytosis.
3 Severe: Leukocyte accumulation scattered across large areas of muscle, or with large multifocal clusters, accompanied by a significant loss of muscle fibre integrity with active phagocytosis and accompanied with or without moderate/severe presence of necrosis and/or fibrosis.

FIBROSIS: Muscle fibrosis is often the end result of an initial inflammatory process. In H&E staining, fibrosis is characterised by increased amounts of pale eosinophilic fibrillar material (collagen deposition) separating and surrounding adjacent myofibers. In the Mallory’s Trichrome stain, collagen fibres appear stained in blue (recommended staining for scoring). Recommended microscope magnification for scoring is 10X. A magnification of 20X may be used for a more detailed view, especially recommended in mild fibrosis.

0 Normal: Normal connective tissue, collagen is present in epimysium, perimysium and endomysium at normal levels.
1 Mild: Early changes consisting of mild increase of deposits of pale eosinophilic material (H&E) or blue-stained fibrillar collagen (Mallory Trichrome) in endomysium and perimysium.
2 Moderate: Moderate increased deposition of endomysial connective tissue separating and surrounding adjacent fibres with attenuation of several muscle fibres.
3 Severe: Marked presence of collagen deposits in large areas with generalised attenuation and loss of tissue architecture and often with presence of necrotic muscle fibres (dark, purple-stained myoblasts).

**Figure 3: Continued.**
Necrosis is histologically characterised by hypereosinophilic (pale) fibres in H&E, or purple-stained myofibers under Mallory’s Trichrome staining (recommended staining). Necrotic myofibers may or may not exhibit concurrent inflammation, fibrosis and dystrophic mineralisation (calcification). Recommended microscope magnification for scoring is 10X. A magnification of 20X may be used for more detailed view, especially recommended when identifying dystrophic mineralisation (mild necrosis).

<table>
<thead>
<tr>
<th>Normal (0)</th>
<th>Mild (1)</th>
<th>Moderate (2)</th>
<th>Severe (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="https://example.com/normal.png" alt="Normal" /></td>
<td><img src="https://example.com/mild.png" alt="Mild" /></td>
<td><img src="https://example.com/moderate.png" alt="Moderate" /></td>
<td><img src="https://example.com/severe.png" alt="Severe" /></td>
</tr>
</tbody>
</table>

**Normal (0)**: Normal appearance of muscle fibres, characterised by presence of peripheral nuclei, and regular polygonal shape.

**Mild (1)**: Necrotic fibres are focal to a small region, affecting only a few myofibres, very often presence dystrophic mineralisation (calcification). Affected fibres can appear vacuolated and fragmented, pale (H&E) or purple (Mallory trichrome), swollen, round, peripheral nuclei are lost, with minor or no phagocytosis.

**Moderate (2)**: Necrotic fibres are localised to one area, affecting only one bundle of myofibres. Affected fibres can appear vacuolated and fragmented, pale (H&E) or purple (Mallory trichrome), swollen, round, peripheral nuclei are lost. It may be accompanied with moderated leukocyte accumulation and/or active phagocytosis surrounding.

**Severe (3)**: Necrotic fibres are localised to more than one focal area, affecting several muscle fibre bundles. It may be accompanied with moderate leukocyte accumulation and/or active phagocytosis surrounding.

(c)

**Figure 3: Continued.**
Regeneration impairment leads to fibre substitution with ectopic tissues including inter-muscle (perimysium) and intra-muscle (endomysium) accumulation of adipocytes. Recommended microscope magnification for scoring is 10X.

- **0 Normal**: Adipose tissue is present in normal sites (e.g., in perimysium surrounding neurovascular bundles, subcutaneous fat surrounded by epimysium).
- **1 Mild**: Scattered accumulation of adipose tissue (in small clusters) in the perimysium and endomysium.
- **2 Moderate**: Large clusters or moderately scattered accumulation of adipose tissue in the perimysium and endomysium. Hint: Adipocyte infiltration is not present in all fields of view at 10X (not counting areas of severe inflammation).
- **3 Severe**: Markedly increased and widely spread large clusters of adipocyte accumulation in the perimysium and endomysium. Hint: Adipocyte infiltration present in all fields of view at 10X.

**Figure 3: Continued.**
FIBRE DEGENERATION/REGENERATION:

Regenerative myofibers are histologically characterised by the presence of centralised nuclei and cytoplasmic basophilia (slight purple stain) due to large amounts of RNA in actively differentiating and growing cells. They can appear near areas of active necrosis or degeneration and leukocyte infiltration, as macrophages play an important role in phagocytising associated debris. Recommended microscope magnification for scoring is 10X. A magnification of 20X may be also used to obtain a more detailed view of areas of regeneration and centralised nuclei (e.g. score 1 and 2).

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal</td>
<td>Normal appearance of muscle fibres, characterised by presence of peripheral nuclei, and regular polygonal shape. Very few myofibers with a central nucleus may be still present.</td>
</tr>
<tr>
<td>1</td>
<td>Late-stage regeneration</td>
<td>Presence of more mature myofibers, morphology and size of myofibers returning to normal appearance and regular shape, but still with large areas of centralised nuclei present. Muscle fibres have lost the basophilic staining of an actively regenerating myotubes and inflammation has resolved.</td>
</tr>
<tr>
<td>2</td>
<td>Early-stage regeneration</td>
<td>Presence of muscle fibres markedly smaller and homogeneous in size. Myofibers show hypertrophic centralised nuclei (enlarged) and cytoplasmic basophilia. Areas of active inflammation (mild to moderate) may still be present in surrounding areas.</td>
</tr>
<tr>
<td>3</td>
<td>Fibre Degeneration</td>
<td>Degeneration can exhibit a variety of microscopic changes including irregular shapes, cell swelling or, shrunken fibres, hypereosinophilia (pale colour) and necrosis. Accompanied with large areas of leukocyte accumulation (severe inflammation) with marked attenuation or disruption of several muscle fibres.</td>
</tr>
</tbody>
</table>
tissue for each of the categories and levels is included. The histopathological parameters assessed included muscle inflammation, fibrosis, necrosis, adipocyte (fat) infiltration, and muscle fiber degeneration/regeneration. Each parameter was independently scored using an analogue scale of 0 to 3 using a grading sheet (see Supplementary Materials). Additional significant observations were scored +1 if present. Finally, a cumulative ischaemia severity score (cISS) for each muscle sample was obtained by the sum of each individual score. We ensured that a range of different score levels (0 to 3) were present in similar frequencies in each of the categories.

2.7. Appraisers. There were 2 male appraisers and 1 female appraiser, who had good experience visualising histological samples prior to performing the scoring. One appraiser is a qualified consultant pathologist (S.O.H), who would have a wide range of experience in scoring histological specimens from many different tissues. The other two appraisers (C.SN and M.C) are scientists who have experience in observing histological specimens in this study area. The same three appraisers were blinded for the study and evaluated each sample independently. In addition, one observer (C.SN) rescored the 70 samples following a 4-month washout period for intraobserver analysis. To ensure that the order of data collection would not influence results, each appraiser evaluated all samples in a random order using a predefined agreement analysis worksheet.

2.8. First Consensus Scoring Meeting. Prior to commencing the study, the three appraisers met to discuss the parameters and criteria of this scoring system. The definitions for each morphological parameter were reviewed and any discrepancy or lack of clarity in the scoring definition was addressed. Several sample slides were examined by the three appraisers and a score was given. This was to ensure that appraisers had some level of agreement prior to commencing the study.

2.9. Final Conclusion Meeting. A meeting between the three appraisers was arranged at the end of the study to discuss the results. As in some cases appraisers differed in their scores, it
was agreed that it was most appropriate to use the median value of the three scores given to each of the individual histological parameters when calculating the cISS. This value was then used to investigate the association between the cISS with a clinical parameter of disease severity such as calf muscle weight and skeletal muscle fiber diameter.

2.10. Statistical Analysis. Kendall’s coefficient of concordance (W) (a nonparametric rank of ordering concordance) was used to measure the degree of association of ordinal assessments made by the three appraisers [19]. Kendall W accounts for the order of the ratings, for instance, a classification of an observation among raters which differs by 2 points is considered more serious than a classification which only differs by 1 point (e.g., severe (score 3) vs. mild (score 1) is considered worse than severe (score 3) vs. moderate (score 2)). A perfect agreement is indicated by values of 1, while no agreement is indicated by values of 0. Intrarater agreement was assessed similarly. Spearman rank-order correlation and linear regression analysis was performed between muscle weights, and these two parameters. We found a statistically significant strong negative relationship between cISS scores and calf muscle weights with r = –0.863 and 95% CI of –0.920 to –0.772 (p ≤ 0.001). A linear regression analysis indicated that there was an indirect linear relationship between these two parameters (R²adj 75.7%, p ≤ 0.001) (Figure 4(a)). In addition, we investigated the relationship between the cISS and the skeletal muscle fiber diameter, as a measure of skeletal muscle regeneration, and found similar results, including a statistically significant string negative relationship with r = –0.855 and 95% CI of –0.892, –0.733 (p ≤ 0.001) and an indirect linear relationship between these two parameters (R²adj 68.10%, p ≤ 0.001) (Figures 4(b) and 4(c)).

3. Results

3.1. Score Tool Reliability: Interrater and Intrarater Variation for Scoring Histopathological Parameters. Interrater reliability was demonstrated on the basis of a significant level of rank-ordering concordance using Kendall’s W [20]. For all the histopathological parameters scored, we estimated intrarater reliability to be significantly high (Table 2). In most cases, disagreements between the three appraisers were no more than 1 point of difference (e.g., one appraiser gave a score of 3 and another appraiser gave a score of 2). Disagreements among appraisers of ≥ 2 points were minimal (Table 2). Complete disagreements (three appraisers completely disagreed in their scores) were also minimal, i.e., agreement among two appraisers was almost 100% in all the cases (Table 2). Necrosis was the parameter with highest % of agreement, but with lower Kendall’s W. This may be due to having more scores that differed more than 1 point (5/70). Apart from the 5 general histopathological findings assessed, other significant histopathological findings were reported if significant. For instance, abnormal haemorrhage was reported in 2/70 samples. Finally, intrarater reliability was also found to be excellent for all the parameters scored, with Kendall’s W of 0.94 or greater (Table 3).

3.2. Validation of Tissue Pathology: Clinical Measure of Disease Severity. Once appraisers scored all the histopathological parameters, the median value for each parameter was calculated. A cISS per muscle sample was calculated by adding up all the individual scores. The cISS score ranged from 0 (score of 0 in all individual parameters) to 15 (score of 3 in all individual parameters). Additional points (+1) were added to the final score for every other significant histopathological finding (i.e., presence of abnormal haemorrhage). This total score was used to investigate the association between cISS and an objective clinical parameter of disease severity. Calf muscle weight can be a relevant parameter of tissue pathology. We, and others, have observed significant muscle mass loss after ischaemic injury, most likely secondary to muscle necrosis and fibrosis [21, 22]. We hypothesised that the degree of muscle ischaemic damage would be correlated with the level of muscle weight loss and the level of muscle regeneration (e.g., skeletal muscle fiber diameter). Spearman rank-order correlation analysis was performed to investigate the relationship between the cISS score and these two parameters. We found a statistically significant strong negative relationship between cISS scores and calf muscle weights with r = –0.863 and 95% CI of –0.920 to –0.772 (p ≤ 0.001). A linear regression analysis indicated that there was an indirect linear relationship between these two parameters (R²adj 75.7%, p ≤ 0.001) (Figure 4(a)). In addition, we investigated the relationship between the cISS and the skeletal muscle fiber diameter, as a measure of skeletal muscle regeneration, and found similar results, including a statistically significant string negative relationship with r = –0.855 and 95% CI of –0.892, –0.733 (p ≤ 0.001) and an indirect linear relationship between these two parameters (R²adj 68.10%, p ≤ 0.001) (Figures 4(b) and 4(c)).

4. Discussion

Ischaemia in skeletal muscle occurs due to insufficient supply of nutrients and oxygen. In patients with peripheral arterial disease (PAD), ischaemia of distal muscles occurs due to the narrowing or occlusion of peripheral arteries due to the build-up of atherosclerotic plaques [23]. Ischaemic calf muscle in PAD patients is characterised by several histopathological changes such as local inflammation, increased fibrosis and inter- and intramuscle adipocyte content, muscle fiber atrophy, and impaired metabolic function, among others [15, 24]. The most severe manifestation of PAD, namely, critical limb ischaemia (CLI), is characterised by rest pain, nonhealing ulcers, gangrene, tissue loss, and death [23]. In the past years, there has been an increased interest in developing novel therapeutic products aiming to improve tissue perfusion and/or restoration of tissue function in these patients [25]. The mouse model of HLI is considered the most clinically relevant preclinical model of PAD, and especially CLI [26], and has been largely used to assess preclinical efficacy of cell therapy products such as mesenchymal stromal cells (MSCs) [27–31]. In most cases, a complete morphologic assessment of tissue using a range of histological techniques is performed for the treatment group comparisons. However, no standardised tools are used for the assessment of the degree of skeletal muscle damage across all these studies and there exists great variation amongst the histological techniques and quantification methods employed. This may impair interstudy comparability. Semiquantitative histopathology scoring systems have been previously used to obtain semiquantitative data from tissue samples [8]. To our knowledge, there are a small number of studies that have described a semiquantitative scoring system as part of their methodology to assess the level of skeletal muscle damage [10–14]. McCormack et al.
Table 2: Kendall’s W analysis reflecting interrater reliability assessment of histopathological features between three appraisers.

<table>
<thead>
<tr>
<th>Histopathological parameters</th>
<th>% complete agreements (95% CI)*</th>
<th>% agreement between 2 appraisals (95% CI)</th>
<th>% disagreement &gt;2 scores (95% CI)</th>
<th>Kendall’s W*</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td>44.3 (32.4, 56.6)</td>
<td>97.1 (90.1, 99.7)</td>
<td>5.7 (1.6, 14.0)</td>
<td>0.923</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>51.4 (39.2, 63.6)</td>
<td>100 (94.9, 100)</td>
<td>0.0 (0.0, 5.1)</td>
<td>0.942</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Necrosis</td>
<td>58.6 (46.2, 70.2)</td>
<td>98.6 (92.3, 100)</td>
<td>7.1 (2.3, 15.9)</td>
<td>0.771</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Degeneration/regeneration</td>
<td>47.1 (35.1, 59.5)</td>
<td>94.3 (86.0, 98.4)</td>
<td>7.1 (2.3, 15.9)</td>
<td>0.863</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Adipocyte infiltration</td>
<td>41.4 (29.8, 53.83)</td>
<td>98.6 (92.3, 100)</td>
<td>5.7 (1.6, 14.0)</td>
<td>0.914</td>
<td>≤0.001</td>
</tr>
</tbody>
</table>

CI: confidence interval; W: Kendall’s coefficient of concordance; *all appraisers’ assessments agree with each other. *p values were calculated based on Kendall’s W scores from complete agreements.

Table 3: Kendall’s W analysis reflecting intrarater reliability assessment of histopathological features by one appraiser after a 4-month washout period.

<table>
<thead>
<tr>
<th>Histopathological parameters</th>
<th>% agreement (95% CI)</th>
<th>Kendall’s W</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammation</td>
<td>82.7 (71.9, 90.8)</td>
<td>0.978</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>85.7 (75.3, 92.9)</td>
<td>0.977</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Necrosis</td>
<td>90.0 (80.5, 95.9)</td>
<td>0.951</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Degeneration/regeneration</td>
<td>77.1 (65.5, 86.3)</td>
<td>0.943</td>
<td>≤0.001</td>
</tr>
<tr>
<td>Adipocyte infiltration</td>
<td>88.6 (78.7, 94.9)</td>
<td>0.977</td>
<td>≤0.001</td>
</tr>
</tbody>
</table>

CI: confidence interval; W: Kendall’s coefficient of concordance.

Figure 4: Continued.
described an absolute injury score (i.e., percentage of injury) calculated by dividing the number of injured myocytes by the total myocytes scored within 15 photographed fields (approximately 1,000 fibers per animal) [13]. While this scoring system has the advantage of providing quantitative data (e.g., ratio) from a tissue, it does not provide information about other important histopathological parameters such as the level of inflammation, fibrosis, or others. Erkanli et al. described a histological damage score tool for histological evaluation of tissue sections based on a severity level (0: normal, 1: mild, 2: moderate, 3: severe) of disorganization and degeneration of muscle fibers and inflammatory cell infiltration [12]. However, no scoring definitions are provided for each category to guide the observer when performing the scoring [12]. This is likely to result in a reduction of intra- and interrater reliability. Carter et al. described a more comprehensive skeletal muscle histopathology scoring system that scores a lesion’s magnitude on an ordinal scale from 0 to 10 [10]. While a score definition is provided for each category to guide the observer during the scoring, each category scores several parameters at once (e.g., the severity of mononuclear cell infiltration, polymorphonuclear cell infiltration, level of fiber necrosis, and presence of hemorrhage). In cases when a tissue has multiple lesions, it is preferable to assign its own appropriate scoring system for each parameter [8]. This approach is more sensitive, and results in higher interrater repeatability. Also, a large number of ordinal scores may cause difficulty or ambiguity during score assignment and is prone to have reduced repeatability [8]. Indeed, Smajović et al. reported a simplified version of the Carter et al. scoring system to include only 4 levels [11]. Finally, Hardy et al. described a morphometric semiquantitative analysis to assess the extent of muscle injury in four different injury models at different timepoints. In this case, a symbol “+” with more or less +’s is given to each morphological parameter depending on the percentage of tissue affected [14].

Here, we have developed and validated a new semiquantitative histopathological scoring tool to assess skeletal muscle damage due to ischaemia with excellent intra- and interrater reliability. We believe our scoring tool has many advantages over the scoring systems described above. We have used the “splitter” approach, where we have assigned a specific score system to different individual parameters (e.g., inflammation, fibrosis, and necrosis), as it is the preferred approach to use when multiple lesions are present in the same tissue [8]. We also used an ordinal scale with a maximum of 4 score levels for each parameter to describe the severity of the lesion, as it has been previously suggested that 4-5 score levels may be optimal for maximizing detection and repeatability [8]. In addition, we have provided a comprehensive description of each score level including representative examples to guide the raters and enhance interobserver repeatability, which is unique in the literature when assessing skeletal muscle damage (Figure 3). One of the advantages of this scoring system is that a cISS can be calculated by addition of all the individual scores. This gives an overview of the level of skeletal muscle ischaemic damage when taking in consideration all the histopathological parameters examined in the sample. In Table 1, we have proposed an overall interpretation of the level of muscle ischaemia damage based on the cISS by providing specific cISS intervals for a “normal,” “mild,” “moderate,” and “severe” muscle ischaemic damage. Also, here we can confirm that there is a similar distribution of each of the proposed levels across all the 70 scored samples, which is considered important when designing a new scoring system (Table 1).

In this study we applied the scoring system to the whole calf muscle cross-section in order to obtain an overall representation of level of ischaemia-induced skeletal muscle damage (Figure 5). The calf muscle consists of three separated muscles, the gastrocnemius, soleus and plantaris muscles, and therefore, this scoring system could also be
applied to the three separated muscles independently if required. We have observed some differences in regard to the injury-repair process across the three muscle types, which may be due to the different composition of myofiber types, and metabolic demands, which can affect the overall injury-repair process. For instance, the gastrocnemius muscle is primarily composed of MyHC2B fibers (glycolytic fibers), more abundant in superficial regions, with some MyHC2A and MyHC2X in deeper regions. The soleus muscle predominantly presents MyHC1 fibers (slow oxidative fibers) in combination with some MyHC2A fibers; and plantaris muscle is composed primarily of MyHC2B fibers with considerable numbers of MyHC2A and MyHC2X fibers [32].

Overall, we found widespread inflammation and fibrosis across the three muscles 28 days after ischaemia injury (Figure 5). In general, the plantaris muscle was largely affected by ischaemia, with severe inflammation, fibrosis, and necrosis always present. The deeper regions of the gastrocnemius were also largely affected by the ischaemia injury. Fibrosis and inflammation were also observed in the soleus muscle although this muscle seemed to be less affected than the other two muscles (e.g., necrotic clusters of fibers were rarely observed in the soleus). Also, abnormal muscle fat infiltration was rarely observed in the soleus muscle and was most commonly observed in the gastrocnemius muscle. Overall, the soleus muscle seems to recover faster from the injury. In contrast, plantaris and gastrocnemius may still present some histological features, such as fat infiltration or some areas of fibrosis even when inflammation is minimal, and regeneration is ensuing. These findings are in concordance with Charles et al who hypothesised that glycolytic muscles (e.g., gastrocnemius) are more prone to ischaemia-reperfusion-induced injury than oxidative skeletal muscles (e.g., soleus) [33]. Oxidative skeletal muscles are characterised by increased mitochondrial content and enhanced antioxidant defences allowing better protection against ischaemia-reperfusion, while the impaired mitochondrial respiration, increased reactive oxygen species (ROS) production and reduced antioxidant defences found in glycolytic gastrocnemius muscle may be key contributors to the injury. The plantaris muscle is also glycolytic and therefore it is also prone to injury.

4.1. How to Use This Tool. We propose that samples should be scored by a minimum of two independent appraisers blinded to the treatments. While adding more appraisers may result in a reduction of the percentage of agreement, the calculation of cISS may become less biased when there are disagreements, as the cISS can be calculated using the median scores among three appraisers. In the cases where two appraisers score the samples, the two appraisers must discuss the disagreements and agree a final score. Furthermore, if the appraisers do not have experience in this study area, we recommend achieving some level of training prior to starting the scoring. The National Toxicology Program (NTP) Nonneoplastic Lesion Atlas is a publicly available web-based resource containing images, terminology, and guidelines for diagnosis of nonneoplastic lesions in rodents [34]. Thuilliez et al. work has compiled a glossary of definitions and pictorial examples of histopathological lesions often observed in skeletal muscle of rodents after intramuscular injection that may guide the researchers when reporting histological findings [35]. Overall, the lowest percentage of agreement for a specific morphological category scored by two selected appraisers was 51% (Table 4). Therefore, we recommend reaching, at least, 50% agreement in scoring each morphological parameter among two selected appraisers prior to commencing the study. This will

Figure 5: Assembled image of a whole cross-sectional area of ischaemic calf skeletal muscle 28 days after ischaemia. (a) Calf muscle cross-section stained with Mallory’s trichrome staining. (b) Calf muscle cross-section stained with H&E. Images were taken with a 10x magnification and assembled to obtain the overall cross-sectional area. Dotted lines highlight approximate edges of plantaris (P), soleus (S), and gastrocnemius (G) muscles. N = tibial nerve. Scale bar is 0.4 mm.
enhance interrater reliability. When reporting the results, we propose to report both, individual scores and cISS. Average scores (median) of the different experimental groups can be then compared using nonparametric statistical tests. Finally, while this is a simple tool that requires the use of two routine histological stains such as H&E and Mallory trichrome stain (Masson’s Trichrome staining is also valid). Nevertheless, we propose that the use of this scoring system can be complemented with other staining and other quantifiable methods that may be relevant to each particular study.

4.2. Limitations. We caution that Kendall’s W does not imply that any particular appraiser is correct or incorrect, simply whether observers agreed or not. We, however, have validated this tool using a clinical measure of disease severity in these mice, such as calf muscle weight. Muscle wasting and weakness is a common symptom in PAD [36]. We and others have observed muscle mass loss after ischaemia in rodent, which is most likely secondary to muscle necrosis and fibrosis and can return to baseline levels with regeneration [21, 22]. Spearman rank-order correlation analysis showed a strong and statistically significant negative relationship between the cISS and calf muscle weight ($r = -0.863$, $p \leq 0.001$). This convincing finding lends credence and scientific merit to our scoring method, which shows a good representation of the pathology of the tissue. Nevertheless, correlation analysis between skeletal muscle weight and cISS must also be done with caution when using other injury models and/or timepoints. Factors such as adipocyte infiltration, extent of fibrosis or oedema (especially at very early timepoint) [37] may influence muscle weight. In this regard, skeletal muscle fiber size is a reliable and reproducible parameter to indicate muscle fiber regeneration after injury. Here, we have investigated the relationship between cISS and skeletal muscle fiber diameter, and our results showed a strong and statistically significant relationship between these two parameters ($r = -0.855$, $p \leq 0.001$).

There are other parameters that must be taken in consideration prior to using this tool, including the endpoint of the study at which muscles are scored, and the animal strain. Our in vivo study endpoint and assessment has been optimised at 28 days after ischaemia surgery. At this timepoint we have observed significant muscle mass loss compared to the nonischaemic limb, and also muscle gain due to regeneration, which allows the treatment group comparisons (unpublished observations, Sanz-Nogués et al.). However, the study endpoint may differ for other studies. In this regard, one should take caution as the severity of lesions can differ across different endpoints. Moreover, the magnitude and the persistence of the lesion may differ across different types of skeletal muscle injury models. Therefore, the timepoint at which the scoring system is applied may vary across different injury models. In addition to this, it is widely acknowledged that there are differences between inbred strains of mice to surgically induced HLI [38–41]. For instance, C57BL/6 mice showed significantly better collateral artery formation and limb perfusion, and less tissue damage than BALB/c mice in response to HLI [39–41]. BALB/c mice have significantly lower expression of vascular endothelial growth factor A (VEGF-A), poor collateral artery formation, reduced limb perfusion, and impaired recovery [39–41], as well as significantly greater myofiber atrophy, greater apoptosis, and attenuated myogenic regulatory gene expression than C57BL/6 mice [38]. In cases when different animal strains and/or study endpoints are utilised, we recommend first evaluating whether the range of lesions present in the samples can be assessed using the lesion severity proposed in this scoring system for each parameter evaluated.

5. Conclusion

Here, we have developed and validated a novel multi-parametric semiquantitative scoring system that can be used to evaluate the level of ischaemia-induced muscle damage with excellent inter- and intrarater reliability. We propose that this tool can be used for treatment comparisons in preclinical animal models such as the HLI mouse model. Nevertheless, we anticipate that the use of this tool can be extended for assessing muscle damage due to other injuries, as the process of muscle repair and regeneration, as well as the histopathological features evaluated here, have been found to be quite similar in other skeletal muscle injury models widely employed to study regeneration, including cardiotoxin, freeze injury, barium chloride, and notexin injury [14, 22, 37]. As publications on validated multi-parametric semiquantitative scoring system for muscle injury are rare, we believe that this article is an important contribution to the very limited database of published scoring systems.

Data Availability

The authors declare that the data supporting the findings of this study are available within the article and its supplementary information files. The raw data generated and analysed during the current study are available in Supplementary Materials.
Ethical Approval

All animal experiments were carried out in compliance with the Directive 2010/63/EU. Ethical approval was granted by the Animal Care Research Ethics Committee (ACREC) at the University of Galway (Ireland) and appropriate individual and project authorizations were granted by the Health Products Regulatory Authority in Ireland (AE19125/P081 and AE19125/P46).

Disclosure

An earlier version of this article was presented as a preprint in Research Square [42] which is available in the following link: https://www.researchsquare.com/article/rs-1972541/v2.

Conflicts of Interest

T.OB is a founder, director, and equity holder in Orbsen Therapeutics Ltd. The other authors declare that they have no conflicts of interest.

Authors’ Contributions

C.SN, M.C, X.Z, and C.A.L carried out animal work, tissue processing, and staining. C.SN, M.C, and S.O.H carried out tissue sample scoring, analysis, and interpretation of results. C.SN performed statistical analysis. C.SN, M.C, S.O.H, X.Z., K.GW, and T.OB conceived the study, participated in the design of the study, and helped to draft the manuscript. The final manuscript was read and approved by all the authors.

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Supplementary Materials

Supplementary Figure 1: a grading sheet template. This template can be used by the researchers when rating histological samples using this semiquantitative scoring system. Supplementary Figure 2: the interrater and intrarater scoring raw data. (Supplementary Materials)

References


