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Babak Shadgan
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Babak Shadgan,a W. Darlene Reid,b R. Luke Harris,c Siavash Jafari,d Scott K. Powers,e and Peter J. O’Brienf

aUniversity of British Columbia Hospital, Unit 1B—Room F329, 2211 Wesbrook Mall, Vancouver, BC, V5Z 1L8, Canada
bVancouver Coastal Health Research Institute (VCHRI), Muscle Biophysics Laboratory and Department of Physical Therapy, 617–828 West 10th Avenue, Vancouver, BC, V5Z 1L8, Canada
cUniversity of Northern British Columbia, School of Health Sciences and Northern Medical Program, T&L #10-3590, 3333 University Way, Prince George British Columbia, BC, V2N 4Z9, Canada
dUniversity of British Columbia, School of Population & Public Health, Vancouver, BC, V6T 1Z3, Canada
eUniversity of Florida, College of Health & Human Performance, Gainesville, Florida 32611-8208
fUniversity of British Columbia, Division of Orthopaedic Trauma, Department of Orthopaedics, 110–828 West 10th Avenue, VCHRI, Vancouver, BC, V5Z 1L8, Canada

Abstract. During orthopedic procedures, the tourniquets used to maintain bloodless surgical fields cause ischemia and then reperfusion (I/R), leading to oxidative muscle injury. Established methods exist neither for monitoring orthopedic I/R nor for predicting the extent of tourniquet-associated oxidative injury. To develop a predictive model for tourniquet-associated oxidative muscle injury, this study combined real-time near-infrared spectroscopy (NIRS) monitoring of I/R with Western blotting (WB) for oxidized proteins. We hypothesized strong correlations between NIRS-derived I/R indices and muscle protein oxidation. In 17 patients undergoing ankle fracture repair, a thigh tourniquet was inflated on the injured limb (300 mmHg). Using a continuous-wave (CW) NIRS setup, oxygenated (O₂Hb), deoxygenated (HHb), and total (tHb) hemoglobin were monitored bilaterally (tourniquet versus control) in leg muscles. Leg muscle biopsies were collected unilaterally (tourniquet side) immediately after tourniquet inflation (pre) and before deflation (post). Average ischemia duration was 43.2 ± 14.6 min. In post-compared to pre-biopsies, muscle protein oxidation (quantified using WB) increased 172.3% ± 145.7% (P < 0.0005). Changes in O₂Hb and tHb were negatively correlated with protein oxidation (respectively: P = 0.040, R² = 0.25 and P = 0.003, R² = 0.58). Reoxygenation rate was positively correlated with protein oxidation (P = 0.041, R² = 0.25). These data indicate that using CW NIRS, it is possible to predict orthopedic tourniquet-associated muscle oxidative injury noninvasively. © 2012 Society of Photo-Optical Instrumentation Engineers (SPIE).[DOI: 10.1117/1.JBO.17.8.081408]

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1 Introduction

Post-surgical complications are common following orthopedic procedures that use limb tourniquets to maintain a bloodless surgical field in the extremities. These complications include muscle paresis, impaired wound healing, infection, compartment syndrome, deep vein thrombosis, and increased frequencies of limb pain, swelling, and neuromuscular dysfunction.1–10

The principal causes of tourniquet-induced neuromuscular dysfunction appear to be direct pressure on the nerve and skeletal muscle directly underlying the tourniquet, muscle and microvascular oxidative injury distal to the tourniquet, and reperfusion-associated oxidative stress and inflammation distal and proximal to the tourniquet.

Among several variables related to tourniquet cuff inflation that apparently contribute to the development of ischemic muscle injury, the duration of ischemia has been identified as the primary factor.4,11 Several studies have reported that, as long as the duration of ischemia does not exceed 3 h, skeletal muscle escapes irreversible damage due to ischemia and the reperfusion that subsequently occurs (I/R) upon tourniquet release.12–19 The safe time limit for tourniquet use in humans remains controversial, however.10,20–22 The current clinical standard for maximum continuous tourniquet time during lower-extremity orthopedic surgery is 90 min.4,6,11,23,24 This benchmark is based primarily on animal studies, but the clinical relevance of such studies is open to question.16,23,25

Surgeons would be better able to balance the advantages of a bloodless operative field against the risks of prolonging muscle recovery and rehabilitation owing to ischemic muscle injury if there were practical, noninvasive monitoring methods that helped determine safe surgical tourniquet time and pressure on a patient-by-patient basis. One potential solution to this problem is near-infrared spectroscopy (NIRS), a noninvasive optical method for the real-time monitoring of tissue oxygenation and hemodynamics.26,27

The science of NIRS hinges on some of the fundamental principles of optics and photonics as they relate to the transmission of light through living tissues and the absorption of light by tissue chromophores. NIRS units use lasers or diodes that...
transmit pulses of multiple wavelengths of light into tissues and optical sensors that detect returning photons. When NIR light is transmitted through tissue, some is irretrievably lost due to scattering and some is absorbed by compounds other than the chromophores of interest. Only a small proportion of the original photons transmitted can be detected returning from the tissue. The changes in absorption at discrete wavelengths generate raw optical data that can be converted by mathematical software algorithms into real-time concentration changes for each chromophore using a modification of the Beer—Lambert law. The principal chromophores of interest in physiological and clinical studies using NIRS are oxygenated \( \text{O}_2\text{Hb} \) and deoxygenated \( \text{HHb} \) species of hemoglobin, which each have a distinct extinction coefficient (absorption characteristic) across the NIR spectrum.

NIRS has been validated and used by many investigators to monitor regional tissue oxygenation, hemodynamics, and metabolism in health and disease. In fact, the use of NIRS for monitoring skeletal muscle hemodynamics during tourniquet use has previously been validated in animals using a rat model. However, the application of NIRS in monitoring human limb muscle ischemia is limited to studies regarding limb muscle oxygenation during short-duration venous and arterial occlusion.

Accordingly, the purpose of this clinical study was to investigate leg muscle oxygenation, hemodynamics, and oxidative injury (measured according to protein oxidation) during orthopedic surgical tourniquet use. Specifically, using NIRS we monitored skeletal muscle oxygenation and hemodynamics distal to the tourniquet during surgery, and then examined ischemia-related muscle biopsy protein oxidation using biochemical Western blotting (WB) analyses. We also aimed to establish the feasibility of NIRS for monitoring clinical muscle ischemia over extended periods and to develop a NIRS-based approach for predicting tourniquet-associated muscle oxidative injury.

We hypothesized that oxidative damage to muscle proteins distal to the tourniquet would be correlated both with longer tourniquet times and with a slower rate of muscle reoxygenation following tourniquet release. We also hypothesized that NIRS would be a feasible method for noninvasive monitoring of prolonged skeletal muscle ischemia in the clinical setting.

2 Materials and Methods
2.1 Participants
A convenience sample of patients with closed ankle fractures requiring emergency surgery was recruited upon admission to a level 1 trauma hospital. Inclusion criteria were adults with unilateral ankle fractures, no major comorbidity, and no additional previous or current injuries to either limb that might affect the reliability of NIRS measurements. The study received institutional clinical research ethics board approval. Informed consent was obtained from all volunteers before their participation. All procedures complied with the Declaration of Helsinki.

2.2 Experimental Overview
All patients received standard general anesthesia. After surgical preparation and positioning of the lower limbs, a pair of NIRS probes (Oxymon M-III, Artinis, the Netherlands), each with 4-cm interoptode distances, which provided a 2-cm penetration depth, were fixed with surgical tape over the tibialis anterior muscle and the peroneus tertius muscle (PT) distal to the tourniquet (1) immediately after tourniquet inflation (pre), and (2) toward the end of surgery, immediately before tourniquet deflation (post). The tourniquet was released when the surgeon no longer required arterial obstruction.

The TA was chosen for NIRS monitoring because it is the most superficial muscle within the anterior compartment of the leg, and it provides sufficient surface area for NIRS probe attachment. During ankle surgery, there is no direct surgical access to the TA, but there is direct access to the PT, which is also in the anterior compartment. So, to minimize the impact on the patient, the PT was selected for surgical biopsy. In addition to their similar anterior compartment location, the TA and PT are comparable in their functionally classified motor unit type distributions, so we assumed that biochemical assays from the PT would serve as appropriate comparisons to NIRS measurements from the adjacent TA.

2.3 Near-Infrared Spectroscopy
Oxygenation and hemodynamics were continuously monitored bilaterally, in both TAs, using a four-channel continuous-wave (CW) near-infrared spectroscopy (Oxymon M-III, Artinis, the Netherlands). The Oxymon is designed as a plug-and-play instrument. It is a continuous-wave NIRS using wavelengths in 780 and 855 nm with a sampling rate of 50 Hz to 10 s. NIRS principles and the calculation of NIRS-derived parameters have been described elsewhere and are summarized...
In this study, we measured changes in O$_2$Hb and HHb chromophore concentrations in the TA muscles throughout surgery, from at least 10 min before tourniquet inflation until O$_2$Hb returned to baseline values following tourniquet deflation. Tourniquet time (duration of tourniquet inflation) and O$_2$Hb and HHb concentration changes, along with several NIRS variables, were calculated for each subject based on the data collected during tourniquet inflation and after deflation. These variables included the following. (1) Total hemoglobin (Hb), the sum of O$_2$Hb and HHb concentrations, demonstrates changes in local blood volume in the tissue being monitored. (2) Hb difference (Hb$_{diff}$), the difference between O$_2$Hb and HHb concentration changes, is used as an index of tissue oxygenation. (3) Reactive hyperemia reveals transient increases in tHb upon reperfusion and is used to evaluate the effect of ischemia on vascular function. (4) Recovery time, the time required for O$_2$Hb to recover to preischemic levels from maximum deoxygenation at the end of the ischemic period, is considered to be an index of tissue oxygen consumption during reperfusion. (E) Reoxygenation rate, the rate of increase in O$_2$Hb concentration during the first 3 sec of reperfusion, is used to evaluate the speed at which recovery starts upon reperfusion, which is linked to microvascular function. To control for possible general measurement errors, simultaneous data were also collected from the same location on the control side. Real-time data were sampled at 10 Hz and recorded by the NIRS instrument for further offline analysis using dedicated software (Oxysoft, Artinis, the Netherlands). Changes in tissue oxygenation, deoxygenation, and local blood volume were estimated from changes in O$_2$Hb, HHb, Hb$_{diff}$, and tHb.

In addition to NIRS monitoring, any tourniquet adjustments or other changes in surgical setup, including limb repositioning, that might alter the NIRS sampling during the experiments were recorded. Furthermore, surgical fields were visually inspected during the operations to check for the possibility of blood loss.

### 2.4 Biopsy Collection and OxyBlot Analysis

Immediately upon collection, biopsies were embedded in freezing medium, frozen in liquid nitrogen-cooled isopentane, and stored at −70°C until processing. Protein oxidation was measured by WB for reactive carbonyl derivatives using the commercially available OxyBlot protein oxidation detection kit, according to the manufacturer’s instructions (Millipore, Billerica, MA) and as previously described. Carbonyl groups of myofibrillar protein side chains were derivatized to 2,4-dinitrophenyldihydrazone (DNPH). DNPH-derivatized protein samples were separated using polyacrylamide gel electrophoresis, after photometric total protein determination of each sample to ensure equal protein loading across lanes. Separated proteins were electrotransferred from the gels to nitrocellulose membranes. Membranes were treated with (1) primary antibodies against DNPH, (2) horseradish peroxidase—conjugated secondary antibodies against the primary antibodies, and (3) chemiluminescence reagents. Images of chemiluminescence-treated membranes were digitally captured and analyzed (GelDoc 2000, BioRad, Hercules, CA). This computerized analysis method background-corrects the chemiluminescence images and then uses the chemiluminescent signal intensity of each lane to measure that lane’s volume of oxidized protein using integrated densitometry. Samples from all subjects were run on both pre and post gels. On each post gel, randomly selected pre samples were run as controls that were used to normalize the post to the pre gels. Thus, pre—post differences in the degree of protein oxidation for each subject were calculated from the background-corrected signal intensities of the pre and post gels using the average, normalized pre—post ratio (pairwise comparisons) of integrated densitometry values.

### 2.5 Statistical Methods

Subject characteristics were summarized using descriptive statistics. Statistical differences in average protein oxidation between the pre and post biopsies were assessed using a paired student’s $t$-test. During ischemia (from tourniquet inflation to deflation) in both experimental and control TAs, statistical differences between O$_2$Hb, HHb, and Hb$_{diff}$ concentration changes were assessed using paired student’s $t$-tests. We then used a linear regression model to investigate the effect of each of the following variables on changes in protein oxidation observed in the comparison of pre and post-biopsy samples: age, sex, body mass index (BMI), tourniquet time, O$_2$Hb, HHb, tHb, Hb$_{diff}$, hyperemia interval, recovery time, and reoxygenation rate. Each of these independent variables was entered individually and sequentially into the simple linear regression model. Data were analyzed using SPSS software (SPSS for Windows, Rel.11.0.1. 2001, SPSS Inc., Chicago, IL). Values are reported as means ± standard deviation. Statistical significance was accepted at $P < 0.05$.

### 3 Results

#### 3.1 Descriptive Characteristics

Seventeen patients (13 women, 4 men) with unilateral ankle fractures were included in this study. The mean participant age was 49 ± 15 (range 19 to 69) years. The mean participant BMI was 25.9 ± 4.4 kg/m$^2$.

#### 3.2 Tourniquet

A tourniquet application pressure of 300 mmHg was maintained in all subjects. A bloodless surgical field was obtained in all subjects. The average tourniquet time (duration) was 43.2 ± 14.6 (range 20.7 to 73.8) min.

#### 3.3 Cardiovascular

During tourniquet application, mean arterial pressure increased 7.5 ± 4.9 mmHg. Pulse rate and SpO$_2$ showed no significant changes.

#### 3.4 Near-Infrared Spectroscopy

During ischemia, TA chromophore concentration changes for O$_2$Hb, HHb, and tHb were significantly different in the experimental leg compared to the control leg ($P < 0.05$) and were similar across subjects. Figure 2 shows HHb, O$_2$Hb, tHb, and Hb$_{diff}$ changes in the TA distal to the tourniquet (left side) compared to the control TA (right side) in a single representative subject before, during, and after thigh tourniquet inflation. After tourniquet inflation in all subjects, the ischemic TA demonstrated a progressive increase in Hb$_{o}$ (23.7 ± 8.2 μM) and a progressive decrease in O$_2$Hb (23.4 ± 8.2 μM). These Hb and O$_2$Hb changes began to reverse immediately after tourniquet deflation. During tourniquet inflation, tHb increased in eight subjects (7.8 ± 5.2 μM) and decreased in nine subjects.
Hbdiff showed a significant decrease (~47.1 ± 13.4 μM) during tourniquet application. In control TAs, no significant changes in NIRS variables were observed before, during, or after tourniquet application.

After tourniquet release, values for recovery time and re-oxygenation rate of hemoglobin were, respectively, 138.7 ± 63.7 sec and 5.8 ± 4.1 μM/sec. The Hbdiff decrease rate was significantly higher among male subjects (P = 0.007).

### 3.5 Muscle Biopsy

On average, 43.2 ± 14.6 min of tourniquet-induced ischemia was associated with a 172.3% ± 145.7% increase in total myofibrillar protein oxidation (P < 0.0005) (Fig. 3). Interestingly, however, there was no statistically measurable relationship observed between tourniquet duration and pre—post biopsy changes (P = 0.49, R² = 0.031).

No correlations were found between pre—post protein oxidation and either the age (P = 0.48, R² = 0.03) or the BMI (P = 0.8, R² = 0.04) of the subjects. We observed statistically significant differences between men and women in the average pre—post protein oxidation increase (not shown: 51% greater increase in men, P = 0.022, R² = 0.30). However, the statistical power for this comparison was low due to the small number of men in our sample (n = 4).

![Fig. 2](https://www.spiedigitallibrary.org/journals/Journal-of-Biomedical-Optics) Chromophore concentration changes for O₂Hb and HHb, and NIRS variables of thb and Hbdiff in the tibialis anterior muscle distal to the tourniquet (left side) compared to the control tibialis anterior muscle (right side) in a single representative subject before, during, and after thigh tourniquet inflation. Changes in Hbdiff (the difference between changes of O₂Hb and HHb concentrations) indicate local tissue oxygenation in the tibialis anterior muscle. A: Tourniquet inflation time; B. Tourniquet release time; C: Point of maximum hyperemia; D: Point of maximum O₂Hb recovery.

![Fig. 3](https://www.spiedigitallibrary.org/journals/Journal-of-Biomedical-Optics) Raw oxidized protein volume in peroneus tertius samples at the beginning (pre) and end (post) of tourniquet inflation. The raw contents of carbonylated proteins in peroneus tertius muscle biopsy homogenates were determined by integrated densitometry of Western blots prepared using the commercially available OxyBlot method. The mean values for pre (white bar) and post (gray bar) samples were calculated after correcting the raw values to the loading control sample that was run on all gels. The percent difference between pre and post (black bar) indicates the increase in protein oxidation that occurred during the ischemic period. As indicated, the pre—post difference was significant at P < 5 × 10⁻⁵.
Linear regression was conducted to assess relationships between each of several NIRS variables and the degree of pre—post protein oxidation. For all subjects, we found that changes in both O$_2$Hb and tHb were significantly negatively correlated with the average increase in protein oxidation. Specifically, linear regressions showed that a 1-μM increase in O$_2$Hb resulted in a 6.1% decrease in the degree of pre—post protein oxidation ($P = 0.04$, $R^2 = 0.25$) (Fig. 4), and a 1-μM increase in tHb resulted in an 11.8% decrease in the degree of pre—post protein oxidation ($P = 0.003$, $R^2 = 0.58$) (Fig. 5). In contrast, for all subjects, reoxygenation rate was significantly positively correlated with the average protein oxidation increase. Specifically, for each unit increase in reoxygenation rate the protein oxidation increase was 18.1% greater ($P = 0.041$, $R^2 = 0.25$) (Fig. 6).

Significant associations were not observed between the average protein oxidation increase and changes in NIRS-derived indices of either hyperemia interval ($P = 0.65$, $R^2 = 0.02$) or O$_2$Hb recovery time ($P = 0.42$, $R^2 = 0.03$). Finally, protein oxidation was not correlated with either HHb or Hbdiff ($P = 0.63$ and $P = 0.31$, respectively).

4 Discussion
We have demonstrated that NIRS is a feasible method for the noninvasive monitoring of skeletal muscle ischemia in the orthopedics setting. Specifically, our data show that tourniquet-induced muscle ischemia lasting 21 to 74 min during lower extremity surgery, without reperfusion, is associated with significant limb muscle protein oxidation. Underlying this association between ischemia and muscle oxidative damage are significant negative correlations between tourniquet-induced muscle protein oxidation and local muscle blood volume changes measured using NIRS. However, our data do not support our hypotheses that during tourniquet-induced lower-extremity ischemia, muscle protein oxidation would be correlated with both (1) longer tourniquet duration and (2) a slower muscle reoxygenation rate after tourniquet release. Rather, we demonstrate that faster muscle reoxygenation is correlated with an increased degree of muscle protein oxidation, which nevertheless emphasizes that muscle injury sustained during prolonged ischemia is linked to altered microvascular function.

Harvey Cushing first introduced the pneumatic tourniquet in 1904, and many subsequent investigations have explored the relationship between tourniquet-induced I/R and skeletal muscle injury. Importantly, our findings demonstrate that, on average, 43 min of tourniquet-induced ischemia leads to significant oxidative muscle tissue damage. This is in contrast to previous studies demonstrating that ischemia alone does not lead to protein carbonylation.22,23,55 Our data complement those of Appell and colleagues,22,54,55 who demonstrated in healthy young subjects undergoing ACL reconstruction that only 15 min of tourniquet-induced ischemia, without reperfusion, causes muscle oxidative damage characterized by myofiber edema and capillary basement membrane thickening.

We were surprised to observe that the large increase in tourniquet-associated muscle protein oxidation we report here...
is not statistically correlated with the average tourniquet duration of 43 min. This finding may be clinically important because it indicates that, at least for tourniquet applications up to 1.25 h, increases in protein oxidation are not determined solely by the tourniquet time. Additional factors that may influence tourniquet-associated muscle protein oxidation include the type and severity of trauma; the length of delay between the initial fracture and surgical repair, since muscle unloading is associated with muscle oxidative stress and protein carbonylation; cigarette smoking that generates systemic inflammation and oxidative stress, including in limb muscles; exercise levels and associated myofiber types; nutritional status and inflammation and sepsis associated with traumatic muscle and bone injury.

Reactive hyperemia and increased local blood volume (tHb) after tourniquet release are well documented. In the control TAs, we observed no muscle tHb changes during surgery, but in the surgical limbs we observed tHb increases in nine patients and tHb decreases in eight patients. These tHb changes during tourniquet use were unexpected observations. The gradual muscle tHb increases observed distal to the tourniquet indicate that in these cases tourniquet pressures were likely insufficient to induce complete limb arterial occlusion. In contrast, the gradual decreases in muscle local blood volume (ΔtHb) observed distal to the tourniquet indicate that there was blood loss from the surgical field in these cases. Regression analyses of NIRS variables demonstrated significant negative relationships between muscle protein oxidation and both ΔtHb and ΔO2Hb. Taken together, these findings suggest that muscle oxidative damage distal to the tourniquet is determined partly by changes in local oxygenated blood volume. This observation may suggest that muscles are in fact protected against ischemic injury (i.e., protein oxidation) when there is arterial leakage into the limb at the tourniquet site, but that oxidative injury is intensified when blood loss distal to the tourniquet, at the surgical field, leads to decreased limb muscle blood volume.

Our data show a negative relationship between ΔtHb and the pre—post increase in contractile protein oxidation, which suggests that using a pneumatic tourniquet pressure that permits a very small arterial leak, rather than completely occluding arterial flow, may protect the muscle tissue distal to the tourniquet against ischemia-associated oxidative injury. Consequently, a possible next step from this study might be to develop a "smart" pneumatic tourniquet integrated with a NIRS system, which would control cuff pressure according to real-time muscle ΔtHb values distal to the tourniquet site. In fact, the actual amount of tourniquet pressure required for complete arterial occlusion varies across individuals because, for a given tourniquet cuff pressure, patients with larger limb circumferences, higher BMIs, or uncontrolled hypertension sometimes do exhibit a small amount of blood leakage at the tourniquet site. Further studies with larger sample sizes, additional methods of tourniquet pressure selection, and preferably control trial designs are required to confirm our observation that there is a negative correlation between muscle oxidative damage and changes in local oxygenated blood volume.

Our data also show that, distal to the tourniquet, the degree of muscle protein oxidation is positively correlated with changes in reoxygenation rate. Previous reports demonstrate that I/R injuries are characterized by inflammation and vascular damage, leading to pooling of the oxygen-carrying red blood cells. We speculate that, during ischemia, this combination of increased vascular permeability and red blood cell pooling contributes to faster reoxygenation during reperfusion, as measured using NIRS. Theoretically, this would explain how the degree of protein oxidation could be linked to the reoxygenation rate observed during reperfusion. Thus, with further study, using NIRS to measure the reoxygenation rate could assist surgeons in predicting the degree of tourniquet-induced muscle protein oxidation and, subsequently, to select the most appropriate therapeutic options that have the potential to attenuate I/R injury and improve recovery following surgery.
Such NIRS-based clinical monitoring of tourniquet ischemia may be particularly important because the muscle contractile protein oxidation we report here is likely to have long-lasting consequences. Specifically, recent in vitro evidence suggests that oxidative damage to muscle contractile proteins dramatically increases these proteins’ susceptibility to irreversible destruction by caspase-3, a protein-degrading enzyme that is activated in skeletal muscle following tourniquet use in humans. Animal models also show that posttourniquet recovery of muscle contractile protein composition requires >6 weeks. Clinically, then, if oxidative protein damage is not mitigated, surgical tourniquet use will likely exacerbate functional deficits and prolong postsurgical recovery.

There are limitations to this study. Although NIRS data have been used extensively for studying muscle oxygenation and hemodynamics, this technique should be considered a noninvasive tool only for estimating changes in muscle oxygenation and blood volume. A possible limitation of the protein oxidation/local blood volume correlation we observed is that it is based on two different muscles: the TA and PT were used to collect NIRS and biopsy data, respectively. The TA has a slightly faster motor unit composition than the PT and so, theoretically, protein oxidation should be greater in the TA than the PT because faster muscles are lower in endogenous antioxidants. Additionally, the small sample size in the current study limits the statistical power for finding significant relationships between muscle protein changes and study variables, including ischemia duration and recovery time. Finally, since our cross-sectional study did not include patient follow-up to assess our participants’ postoperative conditions and their leg muscle rehabilitation, we cannot directly compare tourniquet-associated protein oxidation to patient outcomes. Further investigations will both clarify the long-term consequences of tourniquet use for skeletal muscle and establish clear parameters for using NIRS to predict the extent of tourniquet-associated muscle damage.

In summary, this study establishes NIRS as an advantageous and feasible technique for noninvasive monitoring of muscle oxygenation and hemodynamics in the clinical setting. We also demonstrate that large increases in muscle protein oxidation occur during tourniquet-induced ischemia, even without reperfusion. The findings of this study provide a foundation for future clinical investigations. One such investigation may be the development of a NIRS prototype for continuous monitoring of skeletal muscle oxygenation and hemodynamics during prolonged surgical procedures. Another important follow-up may be to investigate the application of NIRS for the early diagnosis of critical muscle ischemic conditions, such as acute compartment syndrome in high-risk trauma patients (Fig. 7).