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Hemodynamic changes in rat leg muscles during tourniquet-induced ischemia-reperfusion injury observed by near-infrared spectroscopy

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Abstract

In this study, we hypothesized that non-invasive continuous wave near-infrared spectroscopy (CWNIRS) can determine the severity or reversibility of muscle damage due to ischemia/reperfusion (I/R), and the results will be highly correlated with those from physical examination and histological analysis. To test this hypothesis, we performed CWNIRS measurements on two groups of male Sprague-Dawley rats (~400 g) that underwent 2 h (n = 6) or 3 h (n = 7) of pneumatic tourniquet application (TKA). Tissue oxyhemoglobin [HbO_2] and deoxyhemoglobin [Hb] concentration changes were monitored during the 2 h or 3 h of 250 mmHg TKA and for an additional 2 h post-TKA. Rats were euthanized 24 h post-TKA and examined for injury, edema and viability of muscles. Contralateral muscles served as controls for each animal. In both groups, [HbO_2] dropped immediately, then gradually decreased further after TKA and then recovered once the tourniquet was released. However, releasing after 2 h of TKA caused [HbO_2] to overshoot above the baseline during reperfusion while the 3 h group continued to have lower [HbO_2] than baseline. We found a significant correlation between the elapsed time from tourniquet release to the first recovery peak of [HbO_2] and the muscle weight ratio between tourniquet and contralateral limb muscles (R = 0.86). Hemodynamic patterns from non-invasive CWNIRS demonstrated significant differences between 2 h and 3 h I/R. The results demonstrate that CWNIRS may be useful as a non-invasive prognostic tool for conditions involving vascular compromise such as extremity compartment syndrome.

Keywords

ischemia; reperfusion; edema; near-infrared spectroscopy; muscle hemodynamics

1. Introduction

Muscle trauma can result from blunt, crush, blast, penetrating and ischemic injury. Ischemic injury can be caused by vascular trauma, tourniquet application (TKA) or acute compartment syndrome (CS). Following ischemic episodes, additional injury may occur during the reperfusion period. This complex process is collectively termed ischemia/reperfusion (I/R) injury (Gute *et al* 1998, Rubin *et al* 1996). Ischemia/reperfusion injury is a major medical problem. The ability to non-invasively determine the extent and reversibility of I/R injury would be a substantial contribution to the care of injured patients.

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Near-infrared spectroscopy (NIRS) has been applied to study various sites within the body including the brain (Schecklmann *et al* 2008), muscle (Wolf *et al* 2007) and breast (Tromberg *et al* 2000, Cerussi *et al* 2007) by providing information on oxy-, deoxy-, total hemoglobin and tissue oxygen saturation non-invasively. NIRS has also been very actively applied to the study of I/R injury in the kidney (Vaughan *et al* 1995a, Vaughan *et al* 1995b), liver (El-Desoky *et al* 2001, El-Desoky *et al* 1999), skin (Thorniley *et al* 1998) and muscle (Binzoni *et al* 1997, Gentilello *et al* 2001) as well as to the study of compartment syndrome (Giannotti *et al* 2000, Tobias and Hoernschemeyer 2007).

In this study, we hypothesized that hemodynamic changes may differentiate I/R tissue characteristics between 2 h and 3 h I/R injured groups and can be detected by non-invasive continuous wave near-infrared spectroscopy (CWNIRS) measurements. These differences could potentially be used to provide a non-invasive indication of the extent and reversibility of I/R injury. To test this hypothesis, we studied the two groups of animals treated with 2 h versus 3 h of TKA. CWNIRS tissue changes pre- and post-tourniquet-induced I/R injury were assessed in the two groups. We then compared the results of non-invasive CWNIRS optical assessment of I/R injury to standard pathophysiologic and histopathologic measures in a well-established animal TKA injury model to determine the feasibility of this approach for detecting the degree of I/R injury non-invasively.

2. Materials and methods

2.1. Animal model

Thirteen male Sprague-Dawley rats from the colonies of Charles River (Wilmington, MA) were used for the experiment. The average weight of the rats was 404 ± 13 g at the time of the experiment. The rats were divided into two groups depending on the duration of the pneumatic tourniquet applications: 2 h (n = 6) and 3 h (n = 7). All animal protocols were approved by the United States Army Institute of Surgical Research and the University of California at Irvine Animal Care and Use Committee. Animals were housed in a vivarium accredited by the American Association for the Accreditation of Laboratory Animal Care.

All procedures were performed under 1.5–2.5% isoflurane anesthesia, adjusted to maintain a surgical plane. A pneumatic digital tourniquet (DC1.6, Hokanson, WA) attached to a tourniquet regulation system (E20, Hokanson, WA) was placed as proximal as possible around the left upper hind limb and inflated to the pressure of 250 mmHg for 2 or 3 h using the previously described methods (Walters *et al* 2008b). The use of 250 mmHg was based on the pilot studies of orthogonal polarizing spectral imaging (Cytometrics, Bridleways, England) to image blood flow. These studies had shown that 220 mmHg effectively eliminated microvascular flow. An additional 30 mmHg was added to further assure the effectiveness of the ischemia. The choice of 2 and 3 h TKA was based upon the previous study showing that 3 h TKA is the threshold duration which induces permanent muscle damage (Klenerman 1980).

During all measurements, we used the contralateral muscles as controls for laboratory findings including hematoxylin and eosin (H&E) histology and the wet/dry weight ratio. The arterial oxygen saturation and heart rate were continually monitored using a pulse oximeter (8600 V, Nonin, MN). In addition, rat body temperature was maintained physiologic using a warm water bed.

2.2. Continuous wave near-infrared spectroscopy

The description of the CWNIRS system and algorithm has been previously described and details can be found in Kim (2005). Briefly, the CWNIRS system consists of a tungsten–halogen light source (HL 2000, Ocean Optics, FL), a CCD spectrometer (BTC111E, BwTek,

DE) and customized optical fiber guides (1 mm diameter and 3 m length made of low OH silica) from Romack Inc. (Williamsburg, VA). Continuous wave near-infrared light was delivered to the lower hind limb of the tourniquet side using a fiber optic probe, and the light-detecting probe was placed in a transmittance mode to collect light intensities at five wavelengths (732, 758, 805, 840, 880 nm) at every 1 s. If we assume that light scattering does not change and oxygenated hemoglobin ([HbO_2]) and deoxygenated hemoglobin ([Hb]) are the only major molecules in tissues with changing concentrations during the study period that absorb light in the wavelength range from 700 to 900 nm; then equation (1), based on Beer–Lambert's law, can be used to estimate their concentration changes:

$$(\Delta \mathrm{OD})^{\lambda} = (\varepsilon_{\mathrm{Hb}}^{\lambda} \; \varepsilon_{\mathrm{HbO_2}}^{\lambda}) \begin{pmatrix} \Delta[\mathrm{Hb}] \\ \Delta[\mathrm{HbO_2}] \end{pmatrix} L, \tag{1}$$

where ΔOD^{λ} is the change of optical density at wavelength λ, $\varepsilon_{\mathrm{Hb}}^{\lambda}$ and $\varepsilon_{\mathrm{HbO_2}}^{\lambda}$ are the extinction coefficients at wavelength λ for the molar concentrations of [Hb] and [HbO_2], respectively, and *L* is the length of light path through the tissues. Optical path length in a scattering medium *L* has been expressed as the source–detector separation *d* multiplied by a differential path length factor (DPF), i.e. $L = d \times \mathrm{DPF}$ (Delpy *et al* 1988). Since we do not account for scattering effects, the unit of Δ[Hb] and Δ[HbO_2] is mM/DPF.

We have programmed data acquisition and real-time display software using the Labview program (Labview 7.0, National Instrument, TX). Basically, this program acquires the full spectrum of transmitted light (600–1000 nm) every second and collects light intensity values at the five wavelengths mentioned above to calculate changes in [HbO_2] and [Hb] using a modified Beer–Lamberts' law (equation (1)). The calculated changes in [HbO_2] and [Hb] were displayed on the computer in real time.

2.3. Experimental protocol

After 10 min baseline CWNIRS measurement of changes in [HbO_2] and [Hb], 250 mmHg of pressure was applied to the tourniquet to induce ischemia of the leg muscles. Tourniquet pressure was released after 2 h or 3 h of ischemia for each group and the recovery muscle CWNIRS measurements were monitored for an additional 2 h. Rats were euthanized at 24 h post-TKA to examine for injury, edema and viability of muscles per standard protocol (Walters *et al* 2008b). The overall study protocol is shown in figure 1.

2.4. Physiologic factors

Muscles from the tourniquet applied leg and a contralateral leg were excised and weighed at 24 h post-I/R injury. The ratio of wet muscle weight between I/R injured and the contralateral leg (Injury/Control) was determined. Muscles were then dried for 5 days at 50 °C to get a ratio of wet to dry muscle weight which represents the level of edema (Wet/Dry). Next, standard H&E staining was performed to assess the damage and edema in muscles histopathologically. Nitrobule tetrazolium (NBT) staining was also performed to compare the percentage of viable cells between 2 h and 3 h TKA groups.

3. Results

3.1. Defining optical factors

To compare the results from tourniquet injury groups, we measured changes in tissue [HbO_2] as shown in figure 2. First, we obtained the ratio of Δ[HbO_2] between Δ[HbO_2]_I (from the baseline to the end of the ischemia period) and Δ[HbO_2]_R (changes during 2 or 3 h of

reperfusion period) ($\Delta[HbO_2]\_I/\Delta[HbO_2]\_R$). Secondly, we measured the time duration between the end point of ischemia and the first peak of $\Delta[HbO_2]$ during reperfusion (Time_Peak). Thirdly, we fitted $\Delta[HbO_2]$ over time during the first 20 min of reperfusion with an exponential model ($\Delta[HbO_2] = A(1-\exp(-t/\tau))$ where A is a fitting amplitude and t is measurement time). The obtained time constant value (τ) was used as the third optical injury assessment measure.

3.2. Hemodynamics from CWNIRS

As can be seen in figures 3(a) and (c), tissue $[HbO_2]$ decreased immediately in both the 2 and 3 h TKA legs, then continued to gradually decrease further during the tourniquet application period and recovered once the tourniquet was released. The average value of $[HbO_2]$ at the end of TKA from the 2 h TKA group is -0.23 ± 0.08 mM/DPF and the 2 h point $[HbO_2]$ value from the 3 h TKA group is -0.27 ± 0.05 mM/DPF. We did not find a statistical difference between these two groups by using a Student t-test ($p > 0.18$). Tissue [Hb] initially increased from both 2 and 3 h TKA legs, and then very slowly decreased following tourniquet application.

However, significant differences in CWNIRS measurements were observed during the reperfusion phase comparing the 2 h with 3 h TKA groups. While release after 2 h of tourniquet application caused $[HbO_2]$ to overshoot above the original pre-tourniquet baseline during reperfusion, the 3 h TKA group continued to have lower $[HbO_2]$ than the baseline $[HbO_2]$. In addition, the 2 h TKA group showed two distinct peaks of $\Delta[HbO_2]$ during 2 h of reperfusion in a number of animals (though this feature was not found in all cases of the 2 h TKA group). Compared to TKA legs, contralateral legs did not show substantial changes of $[HbO_2]$ and [Hb] during ischemia and reperfusion as shown in figures 3(b) and (d).

3.3. Comparison between 2 h and 3 h I/R injuries

The physiologic and optical measurements were compared between 2 h and 3 h I/R injury groups as shown in figure 4. Both 2 h and 3 h TKA caused increase of muscle wet weight compared to the contralateral leg muscles (Injury/Control) indicative of the occurrence of edema in the tourniquet applied leg (figure 4(a)). The 3 h TKA group showed higher values of the Injury/Control ratio indicative of more severe edema than in the 2 h group ($p = 0.02$). The ratio of wet to dry muscle weight (Wet/Dry) was greater in the 3 h injured group than in the 2 h injured group further supporting evidence of increased edema in the 3 h group. NBT staining results showed trends toward greater muscle cell viability in the 2 h TKA group than in the 3 h group (although not statistically significant). Among these three factors, only the Injury/Control ratio clearly distinguished between the 2 h and 3 h groups.

In marked contrast to the invasive physiologic measurements, all optical measurements showed significant differences in 2 h versus 3 h TKA groups as shown in figure 4(b). Among all pathophysiologic and optical factors, the ratio of $\Delta[HbO_2]$ during ischemia and during reperfusion ($\Delta[HbO_2]\_I/\Delta[HbO_2]\_R$) demonstrated the greatest difference between the 2 h and 3 h TKA groups ($p = 0.001$).

3.4. Histopathological differences

Muscle sections from 2 h and 3 h TKA animals were stained using standard H&E methods. Figure 5 shows representative histological images of 2 h and 3 h I/R injuries. Compared to the 2 h group, 3 h TKA caused a greater muscle damage, interstitial edema and hemorrhage. Figure 6 shows representative images of percentage of viable muscle cells among control, 2 h and 3 h I/R injury groups. It is clear that control muscle cells are all viable while I/R injured muscle cells have lost their viability to variable degrees. The severe loss of viability from 3 h I/R injured muscle cells compared to 2 h I/R injury is evident in this case, but not from all cases ($p = 0.14$).

In figure 7(a), two cases from the 2 h injured group show equal or greater values in Injury/Control factor compared to the 3 h group. The results from the Wet/Dry ratio also revealed that only two cases from the 2 h tourniquet applied group had less edema compared to those from the 3 h TKA group, which is the reason why we did not see a statistically significant difference of the Wet/Dry ratio between the 2 h and 3 h groups (figure 4(a)).

3.5. Correlation between physiologic and optical factors

Although both physiologic and optical factors can differentiate 2 h I/R injury from 3 h I/R injury (figure 4), the degree of injury amongst animals is variable and there were some cases of more severe muscle damage/edema from 2 h injury (figure 7). Optical factors, especially $\Delta[HbO_2]\_I/\Delta[HbO_2]\_R$ and time constant, discriminate more clearly between 2 h and 3 h I/R injuries (figures 7(a) and (b)). With regard to the correlation between physiologic factors and optical factors, Time_Peak correlates best with both Injury/Control and Dry/Wet (figures 7(c) and (d)).

4. Discussion

The most important aspect of this study is that we have clearly demonstrated that three non-invasive CWNIRS optical measurement variables are able to distinguish the extent of I/R TKA injury. All of these optical factors were able to differentiate the 2 h TKA group from the 3 h TKA with statistical significance. However, two optical factors, $\Delta[HbO_2]\_I/\Delta[HbO_2]\_R$ and time constant during 20 min of initial reperfusion, are weakly correlated with the Injury/Control ratio which raises questions about the optimal methods for assessing acute I/R injury.

As shown in this study, there is variability in I/R injury amongst tourniquet-treated animals. Not all the 2 h I/R-injured animals showed less edema/muscle damage than the 3 h group (figure 7) by standard invasive analytical methods. Previous results from Walters *et al* showed that 4 h TKA animals had significantly higher muscle wet mass compared to the 2 h TKA group, indicating that the 4 h TKA group experienced greater edema than the 2 h TKA group (Walters *et al* 2008a). The variability of injury, and difficulty in distinguishing the extent of TKA I/R injury using standard invasive methods, underlines the importance of developing non-invasive tools to predict muscle damage.

Near-infrared spectroscopy can non-invasively monitor tissue oxygenation changes and therefore, it has been applied to the studies of compartment syndrome or ischemia-reperfusion injury. Commercial NIRS systems from companies such as Hutchinson Technology, Johnson & Johnson Medical and NIM have been applied to assess tissue oxygenation changes in the rat kidney during I/R injury (Vaughan *et al* 1995b), in the pig leg muscle during pressure increase (Arbabi *et al* 1999) and in the human leg muscle during exercise (Breit *et al* 1997, Egun *et al* 2002, van den Brand *et al* 2004, 2005), or during calf compression (Gentilello *et al* 2001). Most of these presented only a tissue oxygen saturation (S_tO_2) level change in muscle, and the protocols did not exactly simulate I/R injury. Compared to these previous studies, we have obtained changes of $[HbO_2]$ and [Hb] from muscle every second during 2 h or 3 h of tourniquet-induced I/R injury and the following reperfusion period, and correlated the results from optical measurement to standard *in vitro* pathophysiologic and histopathologic measurements. By doing this, we found that non-invasive optical measurement can be a prognostic tool to predict the level of muscle damage due to I/R injury.

During tourniquet application, we expected that blood volume which can be represented by total hemoglobin concentration [THb] (=[Hb] + $[HbO_2]$) would not change appreciably since both arteries and veins are occluded by 250 mmHg of TKA. However, the results did not follow our expectations, and we observed THb decreased during TKA. This phenomenon may be explained as follows. When 250 mmHg pressure is applied to the pneumatic tourniquet (1.6 ×

9 cm), it squeezes the upper limb and blocks the blood flow on the lower limb. Right after tourniquet application, we observed the swelling of the lower limb possibly due to two reasons. First, rat skin is more distensible than human which makes the rat model harder to be used for studying compartment syndromes. Therefore, the lower limb will be expanded when the relatively large area of pneumatic tourniquet squeezed the upper limb analogous to squeezing one end of a balloon causing the other end to expand. However, the local muscles that are physically restricted by the light source and detector probes cannot be expanded, and thus blood from light interrogating muscles will escape to the surrounding muscles/tissues of the lower limb. Secondly, internal pressure raised during ischemia due to the occurrence of edema will also displace the blood in the muscles between the light source and the detector probes. Therefore, we see a great decrease of both HbO_2 and THb while Hb initially increased and then gradually decreased.

One of our optical factors, $\Delta[HbO_2]\_I/\Delta[HbO_2]\_R$, is a potential indicator of how much arterial blood supply recovers after the tourniquet is released. This value is relatively smaller in the 2 h TKA group and larger in the 3 h TKA group. This suggests that the 3 h TKA group has much less recovery of arterial blood supply during the reperfusion period compared to the 2 h TKA group. Meanwhile, time constant values, obtained from fitting 20 min of $\Delta[HbO_2]$ increase during the initial reperfusion period with an exponential model, are used to represent how fast oxygenated blood from artery reaches the tissues that have been ischemic. The 2 h TKA group was clearly distinguished from the 3 h TKA group by recovery time constants. However, this factor again was weakly correlated with 24 h excised tissue pathophysiologic measures muscle edema/damage.

The Time_Peak value, one of the optical factors, is the duration time between tourniquet release and the first peak of $\Delta[HbO_2]$ recovery during the reperfusion period. Even though we are not certain why the peak occurs during the reperfusion phase, we speculate that the peak(s) are from tourniquet pressure release causing a very rapid supply of arterial blood into a locally vasodilated ischemic limb perfusing with blood that contains mostly oxyhemoglobin to the lower limb while pooled venous blood (deoxyhemoglobin) will escape to the upper limb very quickly. As a result, we see a rapid increase of $[HbO_2]$ and drop of [Hb] right after tourniquet release. However, arterial blood supply soon slows down due to built-up pressure in the lower limb during ischemia (edema) and the 3 h TKA group shows a slower increase of $[HbO_2]$ than that from the 2 h TKA group which may indicate a higher built-up pressure in the 3 h TKA group. Therefore, the Time_Peak value may represent recovery effects during reperfusion and correlates well with the Injury/Control ratio and Wet/Dry ratio. Similar results were shown by Vaughan *et al* that 80 min of ischemia in rat kidney caused significantly delayed recovery of HbO_2 during reperfusion compared to that from 45 min ischemia of kidneys (Vaughan *et al* 1995b). However, they did not correlate these results with histological score.

There are a number of limitations to this study. Our CWNIRS system assumes that the scattering loss is constant and does not account for the potential scattering changes due to tissue edema. A detailed study regarding tissue scattering characteristics during I/R injury is beyond the scope of the current manuscript. Therefore, we proposed three different optical factors and presented them along with physical and histological results from 2 and 3 h tourniquet I/R injury cases in order to show the feasibility of the quick assessment of I/R injury using a simple CWNIRS system. More elaborate optical methodologies such as time-resolved systems and more rigorous model-based investigation of muscle physiology would shed more light in the future.

This study does not entirely reproduce the clinical situation in which tourniquets are used. The muscle insult in this experiment does not include a model of the severe limb trauma as seen clinically, with disruption of both soft tissue and bone as well as the additional insult of I/R

with tourniquet placement. This model is intended to serve as a simple, easily reproducible model of tourniquet-induced skeletal muscle I/R injury. Experiments investigating the combined effect of tissue disruption and I/R are needed. Therefore, further clinical patient studies would be needed before applying the results of using this model to clinical practice.

5. Conclusion

Non-invasive CWNIRS was adapted to monitor hemodynamic changes during ischemia and reperfusion injury to determine feasibility of non-invasively detecting differences between 2 h and 3 h TKA injury. We found that optical CWNIRS variables during TKA could distinguish 2 h TKA from 3 h TKA. Strong correlations between the muscle edema level and optical changes were seen. In light of these results, we propose that CWNIRS may have potential application as a non-invasive diagnostic and prognostic tool for I/R injury in extremities. We speculate that CWNIRS might provide a useful tool to monitor the effectiveness of pharmacological interventions which have a goal of reducing the muscle damage due to I/R injury.

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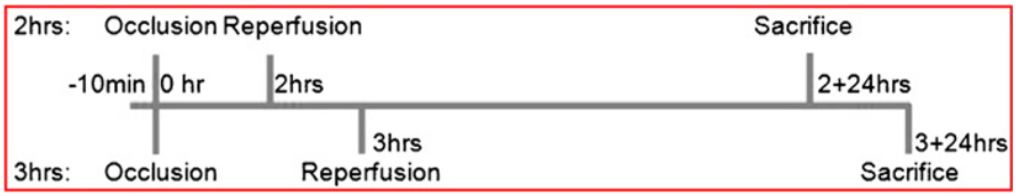

Figure 1.
The study protocol showing either 2 h or 3 h of TKA and sacrifice at 24 h post-ischemia.

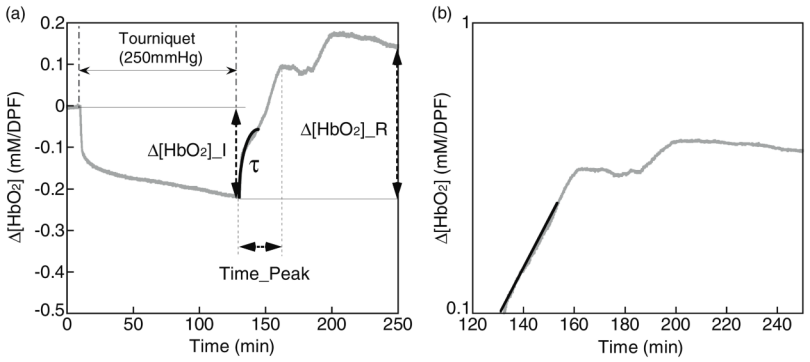

Figure 2.

(a) Optical factors shown on the representative changes in oxyhemoglobin during 2 h of tourniquet application. Three optical factors were defined and correlated with histopathological results: (1) the ratio of $\Delta[HbO_2]\_I$ to $\Delta[HbO_2]\_R$ ($\Delta[HbO_2]\_I/\Delta[HbO_2]\_R$), (2) the time duration between the end point of ischemia and the first peak of $\Delta[HbO_2]$ during reperfusion (Time_Peak) and (3) the time constant value during the initial 20 min of reperfusion (τ). (b) $\Delta[HbO_2]$ during reperfusion plotted in a semi-logarithmic scale.

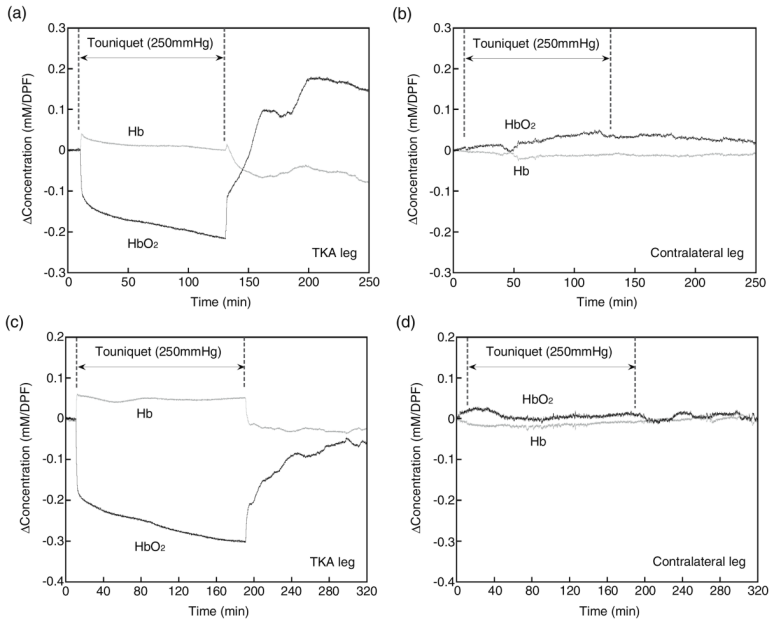

Figure 3.

Representative hemodynamic changes during 2 h TKA (a) and 3 h TKA (c). Contralateral leg hemodynamic changes during 2 h TKA and 3 h TKA from the same animal are shown in (b) and (d), respectively. Compared to TKA legs, contralateral legs did not show substantial changes in hemodynamics during the ischemia/reperfusion period. Significant differences were observed during the reperfusion phase comparing the 2 h with 3 h TKA groups. While release after 2 h of TKA caused [HbO_2] to overshoot above the original pre-tourniquet baseline during reperfusion, the 3 h TKA group continued to have lower [HbO_2] than the baseline [HbO_2]. In addition, the 2 h group showed two distinct peaks of Δ[HbO_2] during the 2 h of reperfusion in a number of animals (though this feature was not found in all cases of the 2 h TKA group).

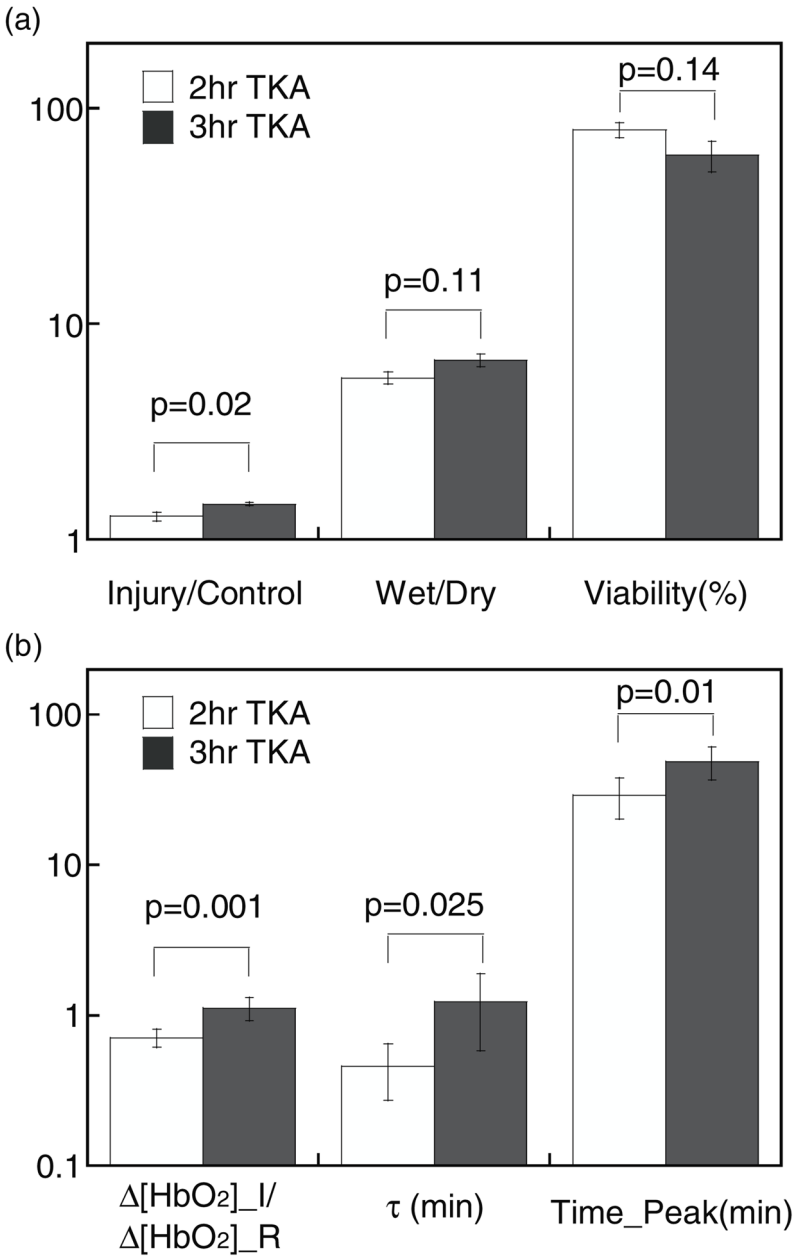

Figure 4.

Physiologic factors (a) and optical factors (b) showing the differences between 2 h and 3 h tourniquet-induced I/R injury. Error bars indicate S.E. and *p*-values from the Student *t*-test. Both physiologic and optical factors could differentiate I/R injury between 2 h and 3 h TKA. Among all pathophysiologic and optical factors, $\Delta[HbO_2]\_I/\Delta[HbO_2]\_R$ demonstrated the greatest difference between the 2 h and 3 h TKA groups ($p = 0.001$).

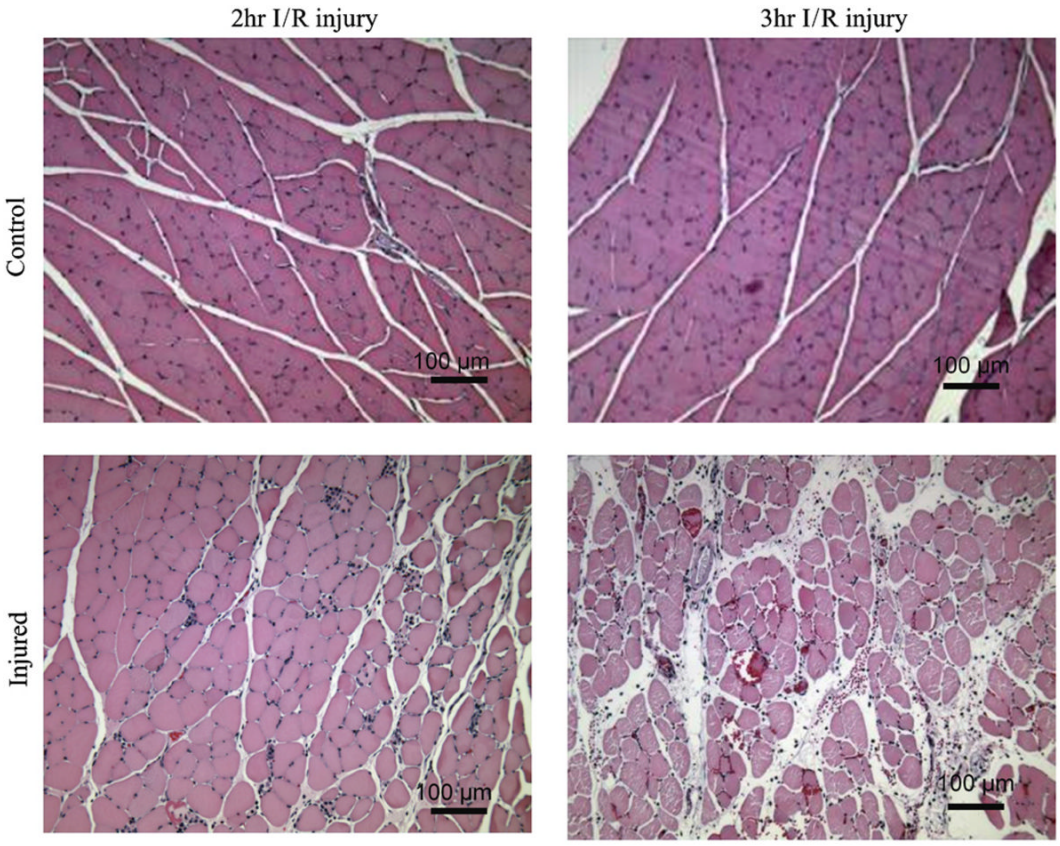

Figure 5.
H&E stained muscle sections from 2 h and 3 h tourniquet-induced I/R injury (×25). Compared to the 2 h group, 3 h TKA caused greater muscle damage, interstitial edema and hemorrhage.

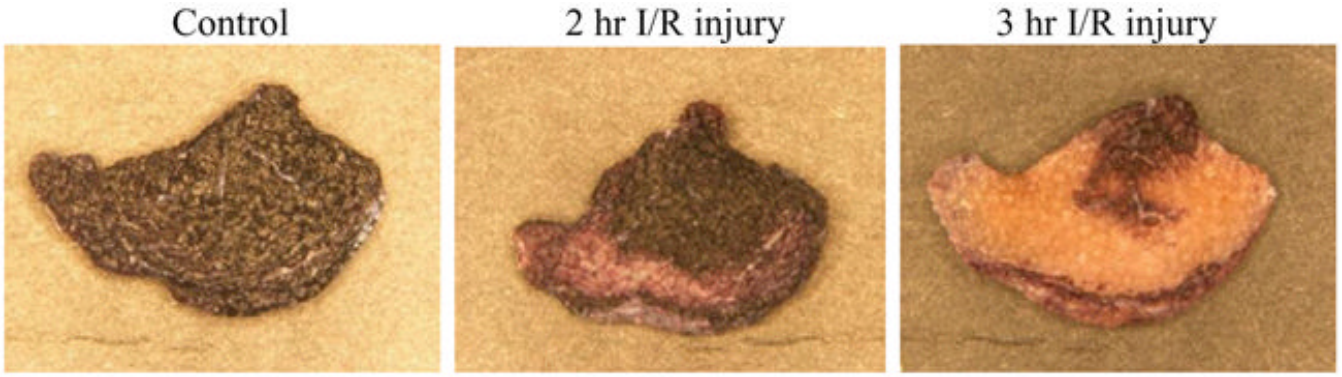

Figure 6.
Muscle section stained with nitroblue tetrazolium (NBT) showing muscle viability. It is clear that control muscle cells are all viable while I/R injured muscle cells lost their viability to variable degrees. The severe loss of viability from 3 h I/R injured muscle cells compared to 2 h I/R injury is evident in this case, but not from all cases ($p = 0.14$).

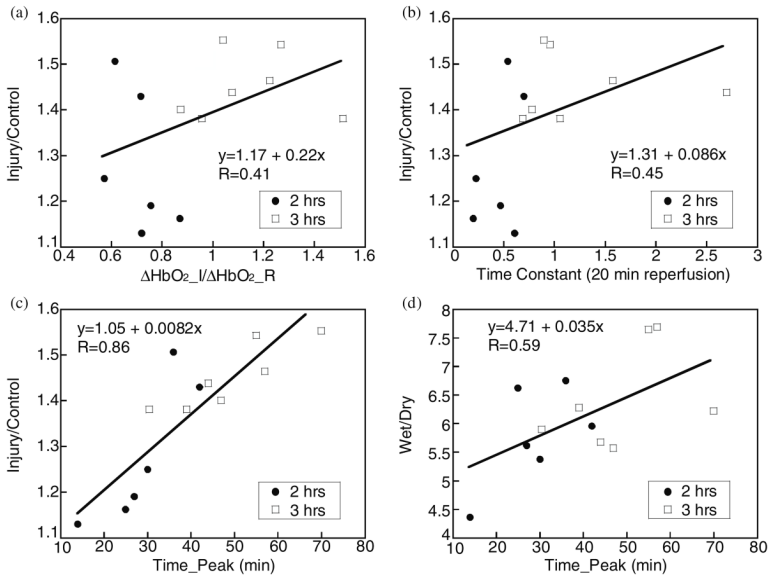

Figure 7.

Plots showing correlations between physiologic and CWNIRS optical variables. The degree of injury amongst animals is variable and there were some cases of more severe muscle damage/edema from 2 h injury. Optical factors discriminate more clearly between 2 h and 3 h I/R injuries. With regard to the correlation between physiologic factors and optical factors, Time_Peak correlates best with both Injury/Control and Dry/Wet.