Cross-section and feasibility study on the non-invasive evaluation of muscle hemodynamic responses in Duchenne muscular dystrophy by using a near-infrared diffuse optical technique

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Abstract: Duchenne muscular dystrophy (DMD) is an X-linked debilitating muscular disease that may decrease nitric oxide (NO) production and lead to functional muscular ischemia. Currently, the 6-minute walk test (6-MWT) and the North Star Ambulatory Assessment (NSAA) are the primary outcome measures in clinical trials, but they are severely limited by the subjective consciousness and mood of patients, and can only be used in older and ambulatory boys. This study proposed using functional near-infrared spectroscopy (fNIRS) to evaluate the dynamic changes in muscle hemodynamic responses (gastrocnemius and forearm muscle) during a 6-MWT and a venous occlusion test (VOT), respectively. Muscle oxygenation of the forearm was evaluated non-invasively before, during and after VOT in all participants (included 30 DMD patients and 30 age-matched healthy controls), while dynamic muscle oxygenation of gastrocnemius muscle during 6-MWT was determined in ambulatory participants (n = 18) and healthy controls (n = 30). The results reveal that impaired muscle oxygenation was observed during 6-MWT in DMD patients that may explain why the DMD patients walked shorter distances than healthy controls. Moreover, the results of VOT implied that worsening muscle function was associated with a lower supply of muscle oxygenation and may provide useful information on the relationship between muscular oxygen consumption and supply for the clinical diagnosis of DMD. Therefore, the method of fNIRS with VOT possesses great potential in future evaluations of DMD patients that implies a good feasibility for clinical application such as for monitoring disease severity of DMD.

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1. Introduction

Duchenne muscular dystrophy (DMD) is an X-linked debilitating muscular disease characterized by progressive muscle weakness and atrophy, the incidence ranged from 1 in 3800 to 1 in 6000 on newborn boys [1, 2]. DMD is caused by mutations in the gene encoding dystrophin, a cytoskeletal protein that provides a physical link between intracellular actin and extracellular matrix [3]. In DMD patients, dystrophin expression is abolished, resulting in reduced sarcolemma stability and rendering the muscle fibers susceptible to contraction-induced injury [4]. As a result, repeated contraction leads to necrosis and regeneration of muscle fibers accompanied by progressive replacement of muscles by fat and fibrotic tissues.

The other important pathogenic mechanism of DMD is that dystrophin deficiency also disrupts the signaling of several dystrophin-associated proteins. Among these proteins, neuronal nitric oxide synthase (nNOS), which is recruited to the sarcolemma by dystrophin and produces the freely diffusible nitric oxide (NO) to the adjacent vasculature to facilitate vasorelaxation and consequently blunt α-adrenergic vasoconstriction during muscle contraction, is particularly important and noticeable [5, 6]. Previous studies in mouse models and patients of DMD have shown that the lack of dystrophin disrupts the recruitment of nNOS to the sarcolemma which decreases NO production and may lead to functional muscular ischemia during exercise [7, 8]. However, the correlation between functional muscular ischemia and clinical phenotype of DMD is still unknown.

In recent decades, standardized multidisciplinary care for DMD has been proposed, including the use of corticosteroids, to prolong ambulation, lessen the risk of scoliosis and to delay pulmonary and cardiac decline [9, 10]. Moreover, several potential therapeutic approaches targeting different pathogenic mechanisms of DMD are currently under investigation, including exon-skipping strategies [11], stop codon readthrough [12], gene repair therapy [13], myostatin inhibition [14], antifibrotic agents [15], as well as vascular-targeted therapies [16]. Coinciding with these advances is the necessity to develop appropriate and non-invasive measures for monitoring disease progression and evaluating the efficacy of potential therapies in clinical trials. Currently, the primary outcome measures in clinical trials have been the functional rating scales including the 6-minute walk test (6-MWT) and the North Star Ambulatory Assessment (NSAA) [17, 18]. Although these
measures are beneficial, they are limited by the subjective consciousness and mood of patients, and can only be used in older and ambulatory boys.

Comparatively, functional near-infrared spectroscopy (fNIRS) reconstructs tissue physiologic parameters based on non-invasive measurement of tissue optical properties such as the absorptions of oxy- and deoxy-hemoglobin. Therefore, fNIRS has been shown to be an effective tool for measuring local changes in tissue oxygenation and perfusion [19]. Additionally, the fNIRS technique has several important strengths such as cost-effective, real-time measurement, long-time monitoring, a highly flexible form of analysis and completely patient-oriented measurement with a time resolution of 1–100 Hz. The fNIRS method of using wavelength range in the optical window (~600-1000 nm) is known can penetrate several centimeters into human tissue. Therefore, the fNIRS method can provide valuable functional insights for various deep tissue measurement applications in health and disease [20–26]. Currently, three major techniques are used in muscle oximetry employing fNIRS [20]. The simplest and most commonly used is continuous wave (CW) technique, based on a constant intensity of light source modulation, which can only provide the relative values of attenuation of light through the tissues. In the frequency domain (FD) technique, the attenuation and the phase delay of light through the tissues can be obtained with an intensity-modulated light source. These two signals of FD technique are related to light absorption and scattering coefficients. In the time domain (TD) techniques, the temporal point spread function (TPSF) of light after propagation through the tissues can be obtained with a picosecond pulse light source for determining the absorption and scattering coefficients of tissue. Therefore, FD and TD techniques can provide the absolute value of the concentration of oxy- and deoxy-hemoglobin. Contrarily, the CW system is solely used to detect changes in absorption coefficient that cannot be used to determine the absolute value of the concentration of oxygenated and deoxygenated hemoglobin. However, the CW system can be miniaturized as the wireless system for long time monitoring and even measurement of freely moving subjects [26]. Additionally, in the clinical studies, the analysis of statistically significant difference before and after some specific test is more important than quantification of absolute value.

Therefore, in the present study, we proposed using the CW-based fNIRS system to evaluate the dynamic changes in muscle oxygenation during a 6-MWT in ambulatory DMD patients. Besides, the venous occlusion test (VOT), which has been introduced for the measurement of tissue hemodynamic responses, in both ambulatory and non-ambulatory DMD patients to investigate whether fNIRS and VOT reflect the severity of the dystrophic process in patients with DMD. Compare with current clinical assessment methods such as 6-MWT and NSAA, the fNIRS measurement with VOT can provide objective and direct physiological information to avoid the limitations of patient's subjective consciousness and mood influence.

2. Materials and methods

2.1 Participants

This study was approved by the Institutional Review Board (IRB) of National Taiwan University Hospital. All participants signed informed consent forms. All experimental methods were conducted in accordance with the latest version of the Declaration of Helsinki. A total of 30 DMD patients were recruited in the joint clinics of neuromuscular disorders in Department of Pediatrics, National Taiwan University Hospital. All patients had clinical presentations consistent with DMD and were diagnosed according to a muscle biopsy with absent dystrophin and/or genetic confirmation of DMD. Demographic data on the patients were collected from the Department of Pediatrics, National Taiwan University Hospital. The DMD patients were classified into two categories, ambulatory (n = 18, mean age 8.28 ± 1.81 years old) and non-ambulatory groups (n = 12, mean age 13.5 ± 2.07 years old), according to their clinical motor function. Furthermore, 30 age- and sex-matched children (mean age 12.77
± 0.68 years old) without a history of weakness or neuromuscular disorders were also recruited as controls.

2.2 Functional near-infrared diffuse optical technique

A continuous wave near-infrared tissue oxygen monitor system (PocketNIRS Duo, DynaSense Inc., Japan) was used to record skeletal muscle oxidative metabolism of all participants. The system includes two optical probes that consist of three light emitting diodes (LEDs) at three wavelengths (735 nm, 810 nm, and 850 nm) and one photodiode (PD) with 3 cm source-detector separation. The time-resolution of detection is up to approximately 60 Hz. 

According to the modified Beer-Lambert Law (MBLL) [27], the optical density (OD) can be defined as follows:

$$OD(\lambda, t) = -\log_{10} \frac{I(\lambda, t)}{I_0(\lambda, t)} = A(\lambda, t) + S(\lambda, t)$$  \hspace{1cm} (1)$$

where $I(\lambda, t)$ and $I_0(\lambda, t)$ are the intensities of incident light and detected light, respectively. The $OD(\lambda, t)$ is the optical density for wavelength $\lambda$ at the time $t$ that means the attenuation of near-infrared light intensity in tissue. The attenuation in tissue is caused primarily by both absorption effect $A(\lambda, t)$ and scattering effect $S(\lambda, t)$ of near-infrared light at a wavelength $\lambda$ at the time $t$. The main absorbers of near-infrared light in tissue are the concentrations of oxy-hemoglobin [HbO2] and deoxy-hemoglobin [Hb]. Therefore, the absorption effect $A(\lambda, t)$ can be defined as follows:

$$A(\lambda, t) = (e_{HbO2}(\lambda)\Delta[HbO2](t) + e_{Hb}(\lambda)\Delta[Hb](t)) \cdot L(\lambda, t)$$ \hspace{1cm} (2)$$

where $e_{HbO2}(\lambda)$ and $e_{Hb}(\lambda)$ are the extinction coefficients of HbO2 and Hb at the wavelength $\lambda$, and $L(\lambda, t)$ is the optical path-length of the wavelength $\lambda$ at the time $t$. In this system, the $L(\lambda, t)$ values include differential path-length factor (DPF) that can be defined as $L(\lambda, t) = DPF(\lambda, t) \cdot d$, where $d$ is the source-detector separation.

Assuming that $S(\lambda, t)$ remains constant during a measurement, the change in optical density can be obtained as follows:

$$\Delta OD(\lambda, t) = OD(\lambda, t) - OD(\lambda, t_0) = -\log_{10} \frac{I(\lambda, t)}{I_0(\lambda, t_0)}$$

$$= (e_{HbO2}(\lambda)\Delta[HbO2] + e_{Hb}(\lambda)\Delta[Hb]) \cdot L(\lambda, t)$$ \hspace{1cm} (3)$$

Therefore, the concentration changes in oxygenated hemoglobin ($\Delta[HbO2]$) and deoxygenated hemoglobin ($\Delta[Hb]$) could be obtained by solving for the MBLL. Then the total hemoglobin ($\Delta[Hb]$) can be calculated by the components of $\Delta[HbO2]$ and $\Delta[Hb]$ (the sum of $\Delta[HbO2]$ and $\Delta[Hb]$). In this system, the optical path-length ($L(\lambda, t)$) was assumed the same value on each wavelength. Besides, the PocketNIRS Duo is a lightweight and portable system with wireless data transmission (Bluetooth). These features are useful for skeletal muscle oxidative metabolism studies of DMD patients such as dynamic changes of muscle oxygenation during the 6-MWT and the VOT.

2.3 Data acquisition

The 6-MWT was performed in ambulatory DMD boys and healthy controls according to the American Thoracic Society (ATS) guidelines accompanying with the measurement of muscle oxygenation of gastrocnemius muscle [28]. Currently, the walking distance of 6-MWT has recommended as a primary outcome measure in ambulatory DMD patients [29–33]. As mentioned before, the distance of 6-MWT is limited by patients’ subjective consciousness and mood, and can only be used in older and ambulatory boys. In this study, the direct
physiological effects of gastrocnemius muscle hemodynamic responses with 6-MWT were recorded in ambulatory participants and controls. Besides, compare with the limitations of 6-MWT, the fNIRS measurement with VOT could be used as an objective potential evaluation method of clinical application for measurement of forearm muscle hemodynamic responses in all participants.

2.3.1 Gastrocnemius muscle hemodynamic responses with 6-MWT

In this protocol, one of the optical probes was placed and fixed on the gastrocnemius muscle by using special ultra-thin double-sided adhesive sheets provided by DynaSense Inc., Japan for dynamic muscle oxygenation detection (as shown in Fig. 1). At each measurement, the probe was cleaned and disinfected with alcohol and a new special ultra-thin double-sided adhesive sheet was replaced.

Fig. 1. Schematic diagram of the experimental procedure of 6-minute walk test (6-MWT): a totally 9 minutes fNIRS measure was performed in three stages: (1) resting-state for 1 minute, (2) 6-MWT, and (3) recovery-state for 2 minutes.

The controller was fixed on the waist and connected the optical probe via 1 m length cable with no loss in signal quality. Before the measurement, the participants were asked to sit on a comfortable chair in a quiet environment with a room temperature of 27°C for 15-20 minutes to ensure have enough rest. After that, a totally 9 minutes fNIRS measurement was performed according to the following three stages: (1) Resting-state (standing still); (2) 6-MWT; and (3) recovery-state (standing still) for evaluating the hemodynamic responses of the gastrocnemius muscle. At first, the participants include ambulatory group and controls were asked to stand still for 1 minute as a period of resting-state. In the stage of 6-MWT, the participants walked back and forth in the area of 20 m that was marked with a tape. Therefore, the walking distances of 6-MWT and tissue hemodynamic responses were recorded to analyse. After 6-MWT, the participants were again asked to stand still at the stop point for 2 minutes as a period of recovery.

2.3.2 Effects of muscle hemodynamic responses with VOT

In this study, the VOT was adopted to evaluate the consumption of muscle oxygenation for all participants as an objective and potential outcome measure. The participants rested in a semirecumbent position, 15-20 min before the measurement. The controllable pneumatic cuff was wrapped around the upper arm with the lower arm at heart level. The optical probe was
placed on the forearm (the area of wrist flexors) and brought in close contact with the skin to prevent noise from the environmental and surface backscattering (as shown in Fig. 2). A totally 4 min fNIRS measurement was performed according to the following three stages: (1) Resting-state; (2) VOT; and (3) recovery for evaluating the change of muscle oxygenation. The baseline measurement was collected at the resting-state period for 1 min. The VOT was carried out with a controllable pneumatic cuff inflated at about 50-60 mmHg on the upper arm, which was maintained for 1 minute before being released. After VOT, the recovery of muscle oxygenation was monitored for 2 minutes. This protocol can be applied to all patients include ambulatory and non-ambulatory DMD patients.

Fig. 2. Schematic diagram of the experimental procedure of venous occlusion test (VOT): a totally 4 minutes fNIRS measure was performed in three stages: (1) resting-state for 1 minute, (2) VOT for 1 minute, and (3) recovery-state for 2 minutes.

2.3.3 Data analysis

The $\Delta[HbO_2]$, $\Delta[Hb]$, and $\Delta[tHb]$ were obtained from fNIRS measurement for analysis of 6-MWT and VOT. Then the average data was obtained for group-level analysis to reduce the effects of individual differences. The results were expressed as the mean ± SE (Standard error). In this study, the slope value (Sv) of $\Delta[HbO_2]$ during VOT was calculated (Be defined as Eq. (4)) for significant differences analysis among the ambulatory, non-ambulatory, and healthy groups.

$$S_v = \frac{\Delta[HbO_2](120s) - \Delta[HbO_2](61s)}{\Delta t = 60s}$$ (4)

The significant difference analysis between healthy ($n = 30$) and DMD groups (All DMD patients $n = 30$) was made with a two-sample $t$-test. As mentioned, the DMD patients were also classified into ambulatory and non-ambulatory groups in this study. Therefore, the non-parametric test was used for analysis of small sample size (sample size $n < 30$). Pairwise comparisons of significant differences (Healthy group v.s. ambulatory group; ambulatory group v.s. non-ambulatory group; healthy group v.s. non-ambulatory group) were made with a Wilcoxon rank sum test. The analyses were performed with MATLAB software (Version R2017b 9.3.0.713579, MathWorks Inc., Natick, MA, U. S.). A more stringent $p$ value of < 0.01 was considered as statistically significant in two-sample $t$-test and Wilcoxon rank sum test.
3. Experimental results

3.1 Relative muscular ischemia during and after 6-MWT in ambulatory DMD boys

Figure 3(a) and (b) show the dynamic change of muscle hemoglobin (relative concentration of $\Delta[HbO_2]$ and $\Delta[Hb]$) of gastrocnemius muscle during 6-MWT. The $\Delta[Hb]$ increased during the stage of standing still in both healthy and ambulatory DMD groups. In the healthy group, the initiation of walking induced decrease in muscle hemoglobin (include both $\Delta[HbO_2]$ and $\Delta[Hb]$) followed by gradually increased muscle oxygenation ($\Delta[HbO_2]$) during a continuation of 6-MWT and obviously increased muscle oxygenation during the stage of recovery (standing still). Contrarily, the exercise-induced initially decreased muscle oxygenation did not increase during the continuation of 6-MWT in the ambulatory DMD group. Additionally, it is noteworthy that although the $\Delta[HbO_2]$ and $\Delta[Hb]$ increased during the stage of recovery in both healthy and ambulatory DMD groups, the $\Delta[HbO_2]$ was lower than $\Delta[Hb]$ in ambulatory DMD group during the stage of recovery, suggesting continuously impaired muscle oxygenation after exercise. In this study, the change of the total hemoglobin ($\Delta[tHb]$) was calculated from the sum of $\Delta[HbO_2]$ and $\Delta[Hb]$. The $\Delta[tHb]$ signal, indicates the local volume of blood in the tissue. Figure 3(c) shows that the $\Delta[tHb]$ was lower in the ambulatory DMD group during all three stages, suggesting less muscular blood supply in DMD patients.

![Graphs showing the effect of 6-MWT on muscle hemoglobin](image-url)
3.2 Impaired muscle microvascular perfusion, reactivity and oxygen consumption during VOT in DMD patients

Because the measurement of 6-MWT has some limitations we mentioned before, thus we applied VOT, which indicates the tissue oxygen consumption, microvascular perfusion, and reactivity, in all subjects including ambulatory and non-ambulatory DMD patients. Figure 4 shows that the dynamic change in muscle oxygenated hemoglobin of forearm was evaluated by using the method of VOT. The results indicate that the variation of muscle oxygenated hemoglobin includes $\Delta[HbO_2]$ and $\Delta[Hb]$ during VOT were significantly different among all three groups.

In the healthy group, the VOT induced apparent increase and adequate in muscle oxygenation (Fig. 4(a)). Contrarily, the muscle oxygenation increased less evident in ambulatory participants, while the muscle oxygenation slightly increased in non-ambulatory participants (Fig. 4(c) and Fig. 4(d)). Besides, the increase in $\Delta[HbO_2]$ was much lower than the increase in $\Delta[Hb]$ in DMD patients (Fig. 4(b), Fig. 4(c) and Fig. 4(d)), while both $\Delta[HbO_2]$ and $\Delta[Hb]$ increase consistently in healthy controls (Fig. 4(a)). Additionally, it is noteworthy that the $\Delta[HbO_2]$ was higher than $\Delta[Hb]$ during the resting and recovery phase in the healthy group. However, in the DMD groups include ambulatory and non-ambulatory, the $\Delta[HbO_2]$ was lower than $\Delta[Hb]$ during the resting and recovery phase, implying relatively muscle ischemia in DMD patients. Overall, the results suggest the impaired muscle microvascular perfusion and reactivity in DMD patients that may provide useful information on the relationship between oxygen consumption and supply for clinical diagnosis.

As mentioned, $\Delta[tHb]$ indicates the local volume of blood in the tissue. Figure 5 shows the dynamic change in muscle $\Delta[tHb]$ that increased during the VOT process. Obviously, the average response of $\Delta[tHb]$ for DMD patients was lower and more slurred than that of the healthy group (Fig. 5(a)), indicating decreased muscle perfusion in DMD patients. There was
also significantly different of \( \Delta [\text{tHb}] \) between ambulatory and non-ambulatory DMD patients (Fig. 5(b)), implying the muscle perfusion deteriorate with progression of disease severity. In conclusion, our results propose that the muscle perfusion is reduced and the microvascular reperfusion and reactivity of muscle are impaired in DMD patients.

### 3.3 Impaired muscle microvascular oxygen consumption and supply in proportion to the functional severity in patients with DMD

According to the result of VOT, the consumption and supply of the muscle oxygenation could be regarded as an important indicator for disease-monitoring of DMD. As shown in the Fig. 4, the VOT induced a more rapid increase in muscle oxygenation in the healthy group. However, the muscle oxygenation increased more slowly during VOT in ambulatory and non-ambulatory DMD groups. To further clarify the relationship between muscle hemodynamic responses and disease severity, the slope of increased \( \Delta [\text{HbO}_2] \) during VOT was calculated based on Eq. (4) for quantitative analysis. Figure 6 shows the statically significant difference of the slope value between healthy and DMD groups (two sample t-test, ** \( p < 0.01 \)).

For further analysis, the slope value of \( \Delta [\text{HbO}_2] \) during VOT in healthy, ambulatory and non-ambulatory groups were demonstrated in Fig. 7. The result shows the significant difference among the three groups. Additionally, the 6-minute walking distance in the healthy group was longer than the ambulatory group of DMD and demonstrated a significant difference between these two groups. The results further support that impaired microvascular consumption and supply correlated with clinical severity in DMD patients.
Fig. 7. The slope of increased Δ[HbO2] during VOT and average walking distance of 6-MWT.

The slope of Δ[HbO2] during VOT is significantly different among healthy, ambulatory DMD and non-ambulatory DMD groups. The average walking distance is also significantly different between healthy and ambulatory DMD groups. (Wilcoxon rank sum test, ** p < 0.01).

4. Discussion

Accumulating evidence has indicated the important role of vascular mechanism in the pathogenesis of DMD. Recent advances in several potential therapeutic strategies for DMD also led to a greater need for objective and non-invasive measures for evaluating disease severity, which correlates with pathological changes. In the present study using fNIRS, we clearly demonstrated the impairment of muscle oxygenation and perfusion during 6-MWT in ambulatory DMD patients. The value of Δ[HbO2] and Δ[Hb] drop at the initiation of walking was thought to be the influences from the stress effect on muscle contraction and sympathetic nerve activity-induced vasoconstriction [34]. We also observed the blood supply of muscle was defective at the stage of recovery of 6-MWT in DMD patients. Furthermore, we showed that the muscle oxygenation and reperfusion decreased with functional severity during VOT in patients with DMD. Taken together, these findings suggest the potential vascular-targeted therapy focusing on vasorelaxation or angiogenesis. Our findings also suggest that fNIRS possesses great potential for evaluating and monitoring disease severity in patients with DMD and the technique may be suitable for inclusion in therapeutic trials.

Compared with normal muscles, which exhibit normal protective mechanisms of α-adrenergic vasoconstriction during muscle contraction, dystrophin-deficient muscles were shown to have defective nNOS-derived NO production, resulting in impaired perfusion and oxygenation of muscles during exercise in mouse models [7]. One previous study also showed impaired functional sympatholysis, which refers to exercise-induced protective attenuation of sympathetic vasoconstriction, in DMD patients [35]. In the present study, we found impaired muscle oxygenation during 6-MWT, which is a primary ambulatory outcome measure in clinical trials, suggesting that functional muscle ischemia may contribute to 6-MWT results in addition to muscle weakness and atrophy in DMD patients. Furthermore, using continuous detection of muscle oxygenation by fNIRS, for the first time, we observed continuously impaired muscle oxygenation after 6-MWT during the recovery phase, implying that the impaired oxygenation of muscles during exercise may have a spillover effect on muscles in DMD patients. In this study, we also observed mildly decreased total hemoglobin concentration, which indicates the local volume of blood in the tissue, at the initial standing state in DMD patients, inferring that the microcirculation of muscle was impaired during both isometric contraction and isotonic contraction. Our finding also suggests fNIRS as a sensitive and objective technique for determining muscle microcirculation of DMD patients.
The fNIRS based on an optical detection for non-invasive and powerful assessment to detect dynamic tissue oxygenation has already been used in various applications including monitoring muscle oxygenation [26, 36–38]. The skeletal muscle is strongly dependent on oxidative metabolism and detection of muscle perfusion and oxygenation is an important parameter in various muscular diseases [39]. However, the skeletal muscle perfusion is relatively low at rest and mainly dependent on capillary blood flow, so that flow measurements in larger vessels are not appropriate to assess the muscle perfusion and oxygenation [40]. Contrast-enhanced ultrasound and perfusion magnetic resonance imaging (MRI) of the muscles have also been used to evaluate muscle microcirculation while ultrasound offers some benefits over MRI because it is inexpensive and time-saving [39, 41]. Compare with these modalities which could only be used at rest, fNIRS provides the superiority for functional exercise studies and can evaluate not only muscle perfusion but also muscle oxygenation [26, 42]. More importantly, the optical method can provide completely patient-oriented measurement. Our results further support that fNIRS is more sensitive in detecting the subtle and functional change in muscle microcirculation and oxygenation.

When the muscle is exposed to the vascular occlusions such as the venous or arterial occlusions, the local hypoxia of the muscle is induced that is related to consumption of muscle oxygenation [42–50]. However, the pneumatic cuff is inflated above 220 mmHg in the arterial occlusion test (AOT) that may run the risk of tissue damage in DMD patients, especially in children. In the study, we applied VOT forearm to evaluate the muscle hemodynamic responses in both ambulatory and non-ambulatory DMD patients. Our results in VOT showed that muscle oxygenation increased adequately with venous occlusion–induced elevation of deoxygenated blood, whereas muscle oxygenation increased less evident during VOT in DMD patients. This result implies that the muscle reperfusion and reactivity are impaired in all DMD patients and further supports that the protective mechanism of nNOS-derived NO production was defective in DMD patients from our 6-MWT finding and previous studies [51]. Considering that the primary ambulatory outcome measure in DMD patients, 6-MWT, is limited by the patient’s subjective consciousness and mood, and can only be applied in older and ambulatory patients, the VOT may provide an objective and reliable measure of impaired functional sympatholysis in muscle [17, 43]. Furthermore, fNIRS study in VOT can be applied easily in younger or non-ambulatory patients with DMD. Further investigation is needed to verify whether the VOT is adequately sensitive for detecting the early impaired muscle oxygenation and reperfusion in very young patients.

Interestingly, we observed lower oxygenated blood and higher deoxygenated blood during resting and recovery phase of VOT in DMD patients. We also found that the total blood volume of muscle during VOT decreased in DMD patients and may deteriorate with progression of disease severity. In combination, our results proposed that the dystrophic muscles are under relatively ischemic condition comparing to normal muscles, which may result from lower muscle hemodynamic responses due to the dystrophic process of muscle accompanied by progressive replacement of muscles with fat and fibrotic tissues in DMD patients [52]. Further studies are warranted to clarify the role of fat effect impaired blood supply in dystrophic muscle.

In the present study, the slope of elevated muscle oxygenation during VOT showed a decreased trend with progression in the DMD stage from ambulatory to non-ambulatory status. Previous studies in the murine model of DMD have demonstrated that the microvascular system of muscle is almost normal in young mice, whereas marked alteration of the microvascular system of muscles was observed in older mice, indicating the disease progression may have a major effect in vascular changes [53]. In brief, accumulating evidence from our and previous studies indicates the importance of early treatment with vascular-targeted therapy in DMD patients. Further studies are needed to elucidate if novel vascular-targeted therapies mainly focusing on vasorelaxation or angiogenesis should be applied to the younger age of DMD patients for better outcome and effects [16, 54].
The present study encountered some limitations. It was performed using a cross-sectional design, and the correlation of muscle oxygenation changes with disease progression and disease severity was not fully explored. Further longitudinal investigations are warranted to clarify whether fNIRS provides a reliable measurement and correlation with disease severity and progression in DMD.

5. Conclusion
To the best of our knowledge, this is the first feasibility study to evaluate muscle hemodynamic responses during 6-MWT and VOT by using fNIRS in patients with DMD. The results clearly demonstrated that the muscle hemodynamic responses decreased with functional severity in patients with DMD, suggesting that vascular-targeted therapies mainly focusing on vasorelaxation or angiogenesis should be applied to the younger age of DMD patients for better outcome and effects. Our results shed light on a novel therapeutic strategy to improve the outcome of DMD. Non-invasive continuous monitoring of dynamic changes in muscle hemodynamic responses by using fNIRS is feasible in healthy and DMD children. This optical technique with a good temporal resolution enables the measurement of muscle hemodynamic responses in muscular small blood vessels where NO acts, which is an important factor in muscular ischemia of patients with DMD during an exercise. The fNIRS measurement with VOT was also shown to be a sensitive and objective technique for assessing DMD severity. From a therapeutic perspective, the application of fNIRS in DMD may be suitable for inclusion in future clinical and therapeutic trials.

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Disclosures
The authors declare that there are no conflicts of interest related to this article.