Clinical and ^{31}P magnetic resonance spectroscopy characterization of patients with critical limb ischemia before and after autologous cell therapy

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Abstract

Autologous cell therapy (ACT) is a new treatment method for diabetic patients with critical limb ischemia (CLI) not eligible for standard revascularization. After intramuscular injection of bone marrow-derived mononuclear cells local arteriogenesis in the ischemic tissue occurs. Studies assessing visualization of this therapeutic vasculogenesis after ACT by novel imaging techniques are lacking. The aim of our study was to assess the effect of ACT on possible metabolic changes and perfusion of critically ischemic limbs using ^{31}P magnetic resonance spectroscopy (^{31}P MRS) and its possible correlation with changes of transcutaneous oxygen pressure ($TcPO_2$). Twenty-one patients with diabetes and no-option CLI treated by ACT in our foot clinic over 8 years were included in the study. $TcPO_2$ as well as rest (phosphocreatine, adenosine triphosphate and inorganic phosphate) and dynamic (mitochondrial capacity and phosphocreatine recovery time) ^{31}P-MRS parameters were evaluated at baseline and 3 months after cell treatment. $TcPO_2$ increased significantly after 3 months compared with baseline (from 22.4 ± 8.2 to 37.6 ± 13.3 mm Hg, p = 0.0002). Rest and dynamic ^{31}P MRS parameters were not significantly different after ACT in comparison with baseline values.

Our study showed a significant increase of $TcPO_2$ on the dorsum of the foot after ACT. We did not observe any changes of rest or dynamic ^{31}P MRS parameters in the area of the proximal calf where the cell suspension has been injected into.

Key words: critical limb ischemia, autologous cell therapy, magnetic resonance spectroscopy

Abbreviations:

ACT – autologous cell therapy

^{31}P MRS – ^{31}P magnetic resonance spectroscopy

$TcPO_2$ – transcutaneous oxygen pressure

CLI – critical limb ischemia

PCr – phosphocreatine

ATP – adenosine triphosphate

Pi – inorganic phosphate

Q_{max} – parameter of mitochondrial capacity

τ_{PCr} – phosphocreatine recovery time

Introduction

Critical limb ischemia (CLI) defined as rest pain and/or gangrene with objectively proven obstruction of lower limb arteries is one of the leading causes of lower limb loss worldwide (Uccioli, et al. 2018). The distribution of CLI in diabetic patients is different from that individuals without diabetes – it affects mainly below-the-knee arteries, is more diffuse and diabetic patients are prone to hypercoagulation and often suffer from lower rates of collateral vessel formation (Vrsalovic, et al. 2017). In accordance with published data, diabetic patients with CLI have 1-year mortality over 25% and the incidence of major amputation is 40% (Norgren, et al. 2007). Due to the distal location of the atherosclerotic lesions many diabetic patients are ineligible for vascular bypass and the primary and secondary limb salvage rates after percutaneous transluminal angioplasty (PTA) are significantly worse compared with non-diabetic individuals (Meloni, et al. 2018,Tenna, et al. 2014). Autologous cell therapy (ACT) by bone marrow-derived mononuclear cells (BMMNCs) is novel alternative technique for patients not eligible for any of standard revascularization procedures. This therapy has been used in clinical trials since 2002 and consistent with latest meta-analyses as well as our own results has been shown to improve parameters of ischemia – i.e. ankle-brachial index (ABI), transcutaneous oxygen pressure ($TcPO_2$) and rest pain scores while also preventing, in most of the studies major amputation (Dubsky, et al. 2017,Lopes, et al. 2018,Rigato, et al. 2017,Xie, et al. 2018).

Some trials have attempted to document formation of new collateral vessels after ACT using a variety of imaging techniques (Ruiz-Salmeron, et al. 2011,Tateishi-Yuyama, et al. 2002). Digital subtraction angiography is too invasive and depends heavily on the amount and speed of contrast instillation as well as on the experience of the interventional radiologist. A computed tomography (CT) angiogram is affected by medial sclerosis and calcification of the vessel wall very prevalent in most of diabetic patients; it also negatively affects kidney

function in patients in higher stages of chronic kidney disease (Pomposelli 2010). $^{99m\text{-}Tc}$ perfusion scintigraphy seemed to be a promising method for assessing the effect of ACT, but we did not confirm any changes of the scintigraphic parameters at rest and after exercise in our recent trial (Nemcova, et al. 2018).

^{31}P magnetic resonance spectroscopy (^{31}P MRS) is a noninvasive technique to evaluate metabolism of muscle by *in vivo* measurement of phosphorus metabolites (PCr – phosphocreatine, ATP – adenosine triphosphate, Pi – inorganic phosphate) involved in muscle bioenergetics. Intracellular pH can be calculated from the difference of chemical shift position signals of PCr and Pi (Moon and Richards 1973). Needless to say measurement of ^{31}P metabolites is associated with physical stress. During physical stress, whereby dynamic changes of signal intensities of PCr, Pi and intracellular pH in muscle are monitored. Parameters as mitochondrial capacity (Q_{max}) and PCr recovery time (τ_{PCr}) can be calculated to help to quantify the oxidative potential of muscle tissue (Kemp, et al. 2001,Valkovic, et al. 2017).

In our previous study we showed significant changes of dynamic MRS parameters in CLI patients in comparison with healthy controls (Sedivy, et al. 2018), therefore we assume that ^{31}P MRS can distinguish different stages of chronic CLI. In the present one, we used both rest and dynamic ^{31}P MRS parameters to assess the effect of ACT on calf muscle energy metabolism. We compared these parameters with changes of transcutaneous oxygen pressure ($TcPO_2$) on the dorsum of the foot. To our knowledge, there are no published data describing ^{31}P MRS assessment of limb muscles during ACT treatment.

Research design and methods

Twenty-one diabetic patients with CLI after unsuccessful standard revascularization, who were treated with ACT in our foot clinic over or 8 years were included in the study.
The clinical effect of ACT was assessed by changes in $TcPO_2$ evaluated at 3 months after cell treatment.

CLI was defined as the presence of non-healing ulcers or gangrene with objectively proven arterial occlusive disease. All patients had Rutherford category 4-6, PEDIS stage 3 with $TcPO_2 < 30$ mm Hg or ABI < 0.6 (Schaper 2004) and all wounds were between stages 2C-3D in accordance with the TEXAS diabetic foot classification (Armstrong, et al. 1998).

The study was approved by the local ethics committee and patients treated by ACT gave written informed consent to undergo the study protocol including detailed oncological, hematological and cardiovascular screening before enrollment as described previously (Dubsky, et al. 2013).

During follow-up, all patients were treated in our foot clinic and received comprehensive therapy of diabetic foot disease. Exclusion criteria for ACT were: diagnosed tumour in any organ, myocardial infarction, stroke or deep vein thrombosis within the preceding 6 months, severe limb oedema, severe hematologic abnormalities and progressive retinopathy with a high risk of eye bleeding.

Cell separation and injection

Bone marrow was harvested from the iliac crest by the Jamshidi technique in the operating theatre and BMMNCs were separated by the use of Smart PReP2 device (Harvest Technologies Corporation, USA) or sedimented using succinated gelatine (Gelofusine, B Braun, Germany) (Sykova, et al. 2006). Final cell suspension of 40-70 mL was injected into the muscles of the affected lower limb in a series of 40-50 punctures of about 1-2 mL each and also to the edges of the wound (Figure 1).

$TcPO_2$ measurement

$TcPO_2$ was measured by the use of using a TCM400 transcutaneous monitor (RadioMeter, city, California, USA). Measurement was done at the dorsum of the foot between the 1st and 2nd metatarsal heads in our foot clinic in a room with standardized temperature with the patient lying supine for approximately 40 minutes.

Rest and dynamic ^{31}P MRS

Magnetic resonance examinations were performed using a whole-body 3T MR system TRIO (Siemens, Erlangen, Germany) with a 11 cm dual-channel $^{1}H/^{31}P$ surface coil (Rapid Biomedical, Rimpar, Germany) and an MR compatible home-made pedal ergometer. All patients underwent ^{31}P MRS of the gastrocnemius muscle in the supine position with the coil fixed under the calf muscles above the wound (Sedivy, et al. 2015). ^{1}H MR imaging to verify the position of the coil was done. Homogeneity of the magnetic field was adjusted by manual shimming on the total proton water signal. After shimming one resting relaxed ^{31}P MR spectrum was acquired by a free-induction decay (FID) sequence with the following parameters: repetition time 15 seconds, 16 acquisitions, sequence length 4 minutes, flip angle 90°. Dynamic spectroscopy (exercise on MR ergometer) was performed only if the patient's physical condition allowed so. Exercise consisted up to 6 minutes (according to actual health condition) of plantar flexions every 2 seconds with a fixed low-level workload of 7 kg. A non-localized FID sequence with a repetition time of 2 seconds was used for dynamic examination. Motion of patients on ergometer was triggered by optic signalization.^{31}P MR spectra were analyzed by the AMARES time domain fitting routine (prior knowledge) in the jMRUI 5.0 software package (jMRUI Consortium). Lorentzian line shapes were used for spectra fitting. Intracellular pH was calculated by Henderson-Hasselbalch equation according

to Moon and Richards (Moon and Richards 1973). Integral values of PCr and Pi were normalised to βATP signal with reference concentration ATP of 8.2 mM in the muscle tissue (Kemp, et al. 2007,Taylor, et al. 1986). Recovery time PCr (τ_{PCr}) was obtained by fitting recovery period PCr data using exponential function. Mitochondrial capacity was calculated according to ADP model (details of the evaluation Q_{max} in (Sedivy, et al. 2015)).

All data were expressed as mean ± standard deviation. Statistical significance was analyzed using paired t-tests. The Shapiro-Wilk Normality Test was done to evaluate normality of ^{31}P MRS parameters. If the condition of normality was not met, the exact sign test was used. A level of $p < 0.05$ was considered statistically significant. The Bonferroni correction to multiple comparisons reduced P values to 0.004 (11 rest and dynamic parameters and $TcPO_2$ were evaluated). Correlation changes of $TcPO_2$ and ^{31}P MRS parameters were assessed using Spearman correlation coefficients.

Results

We examined 21 patients with critical limb ischemia before and 3 months after ACT using ^{1}H MRI (Figure 2A with transversal image of calf muscle) and rest ^{31}P MRS; 12 patients were also successfully examined by dynamic ^{31}P MRS - see example of ^{31}P MRS spectra from rest – exercise – recovery period of dynamic ^{31}P MRS in Figure 2B and example of a PCr record from examination of a patient before and after ACT in Figure 3.

$TcPO_2$ increased significantly after 3 months compared with baseline (from 22.4 ± 8.2 to 37.6 ± 13.3 mmHg; $p = 0.0002$). Results of $TcPO_2$ and rest and dynamic ^{31}P MRS are summarized in Table 1 and Figure 4. Three patients had extremely prolonged recovery of PCr (τ_{PCr} 200>s) and in Table 1 are shown both values τ_{PCr} with and without extremes, resp.

Results of correlation analysis are shown in Table 2. No significant changes of ^{31}P MRS rest or dynamic parameters by ACT treatment were found (only a trend towards a decrease in pH after ACT). Correlation analysis likewise did not show any positive correlation between increasing $TcPO_2$ and change in any of the ^{31}P MRS parameters.

Discussion

The expected effect of ACT is an improvement of limb perfusion and metabolic profiles in the lesion due to the presence of delivered cells. In our patients, this effect is suggested by a significant increase of $TcPO_2$. On the other hand, we did not prove any positive or negative change of rest or dynamic ^{31}P MRS parameters before and after intramuscular injection of BMMNCs in the area of calf muscles above the ulcer.

The absence of any changes in ^{31}P MRS parameters is a positive finding suggesting as it is that muscle metabolism is not impaired by ACT. Moreover, we can assume that ACT stabilizes muscle metabolism at least over 3-month period although we cannot confirm it in comparison with control group. There are several reasons for the absence of positive significant changes of muscle metabolism (only a mild trend in decrease pH at rest) after ACT or for no correlation with $TcPO_2$. The ^{31}P MRS measurement was done in the proximal parts of the calf muscle (deep muscle measurement) whereas $TcPO_2$ was measured on the standardized spot between the first and second metatarsal heads on the dorsum of the affected foot (superficial measurement of skin perfusion) (Fagher, et al. 2018). The co-localization of $TcPO_2$ and ^{31}P MRS measurement is possible – both methods can be done in the middle or proximal parts of the calf muscle, whereas it makes no clinical sense to measure $TcPO_2$ just below the knee (chronic ulcers and gangrene are usually located in the distal part of the foot therefore we measure the microcirculation by the $TcPO_2$ as distally as possible).

Unlike other non-invasive vascular assessment parameters such as ankle-brachial index, $TcPO_2$ is not influenced by medial sclerosis and measures microcirculation by oxygen diffusion through skin; it is considered a standard method for non-invasive measurement of limb ischemia, especially in diabetic patients with CLI. Redlich proved that $TcPO_2$ was a valid predictor for limb salvage in diabetic patients with CLI after below-the-knee PTA, even when angiographic outcome criteria have failed (Redlich, et al. 2011).

Dynamic ^{31}P MRS measures muscle perfusion indirectly – PCr recovery and Q_{max} depend on mitochondrial density and function of mitochondrial enzymes and transporters. The rest PCr/Pi ratio is a general marker of rest cell energy homeostasis not directly related to muscle perfusion. Another reason for the lack of positive results could be the fact that cell metabolism recovery is a longer process compared with ischemic tissue revascularization. After revascularization and formation of new capillaries and collaterals the muscle cells have to increase their vitality before they can utilize the increased blood flow (Asperio, et al. 2001,Morikawa, et al. 1991). Very long τ_{PCr} of the order 200s is not common in published studies. However, our patients were diagnosed with the most severe stage of critical limb ischemia and with very slow recovery of PCr. We assume that these patients are not usually examined by dynamic ^{31}P MRS and if one or two patients had such a high τ_{PCr} in some studies, they were obviously excluded as outliers. In our group only three patients had very high τ_{PCr} values, but we did not exclude them from results because we used pair comparison. Even after the exclusion of these extreme values, the τ_{PCr} parameter was not significantly different before and after ACT.

Most of our patients had atrophic muscles because of lack of movement – they usually offloaded the ulcerated ischemic limb or even used a wheelchair to move. Even after the improvement of ischemia (documented by increased $TcPO_2$), the atrophied muscle cells may not recover enough which could possibly explain the absence of a change in ^{31}P MRS

parameters. Slow muscle fibers continue to atrophy (Figure 2A) with only fast fibers (utilizing anaerobic glycolysis) remaining. Due to excessive pain, limited joint mobility, deformities, contractures or myopathy (caused by ischemia and diabetic neuropathy), patients were usually unable to perform exercise at all or they did it every measurement differently – all those factors could negatively impact the measured ^{31}P MRS parameters. On the other hand, we observed a decrease in pH after ACT which could indicate restoration of the acid-base balance in muscle cells after the treatment. In our previous study, we proved that patients in the most severe stage of limb ischemia had significantly higher pH at rest compared with patients diagnosed with milder limb ischemia and with healthy controls (Sedivy, et al. 2018). The change of rest pH could be explained by the shift of the rest creatine-kinase equilibrium after cell therapy with the increase of $TcPO_2$ – (patients have higher Pi and lower PCr and the creatinekinase reaction consumes H^+) (Wahl, et al. 1994). One of the other reasons of pH change could be cell damage repair – intracellular pH is lower compared with extracellular pH and damaged cells are possibly not able to maintain their homeostasis. Other possible explanation of this paradoxical finding was an adaptive mechanism of the muscle against enhanced production H^+ during workout from anaerobic glycolysis. Intracellular pH reflects the amount of hydrolyzed ATP in myofibrils during muscle workout and, also, mitochondrial ATP synthesis.

Conclusion

Our study showed a significant increase of $TcPO_2$ without documenting any relevant deterioration of rest or dynamic ^{31}P MRS parameters in calf muscles above the wound after ACT compared with baseline and, also, no correlation with $TcPO_2$ values. Results of ^{31}P MRS proved that ACT had no impact on muscle metabolism in the area involved in ACT injection 3 months after the therapy.

Acknowledgements

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No potential conflicts of interest relevant to this article were reported. We declare that results presented in this paper have not been published previously in whole or part, except in abstract format.

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Figures

Figure 1. Injection of cell suspension into the lower limb muscles in a series of 40-50 punctures.

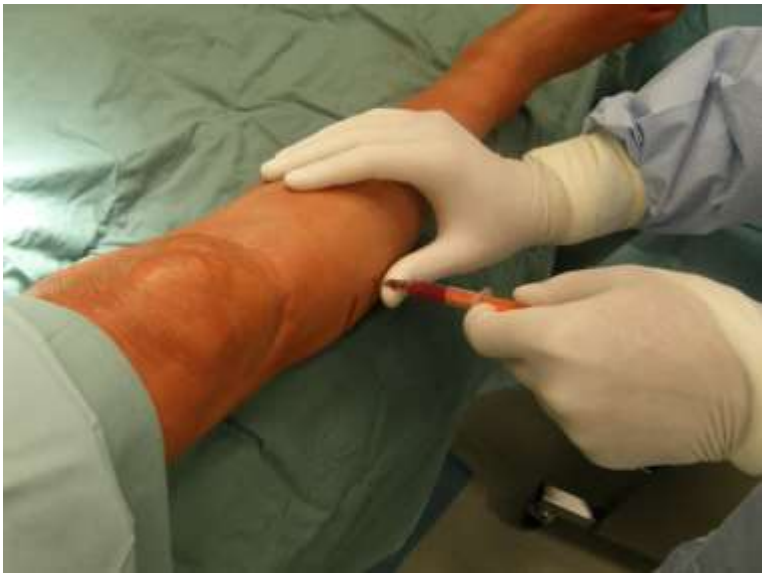


Figure 2. A – transversal MR images of atrophic calf muscle of ischemic patient; B – ^{31}P MR spectra from rest (green), exercise (blue) and recovery (red) period of dynamic ^{31}P MRS. Arrows indicate changes of PCr and Pi during exercise and recovery part of the examination.

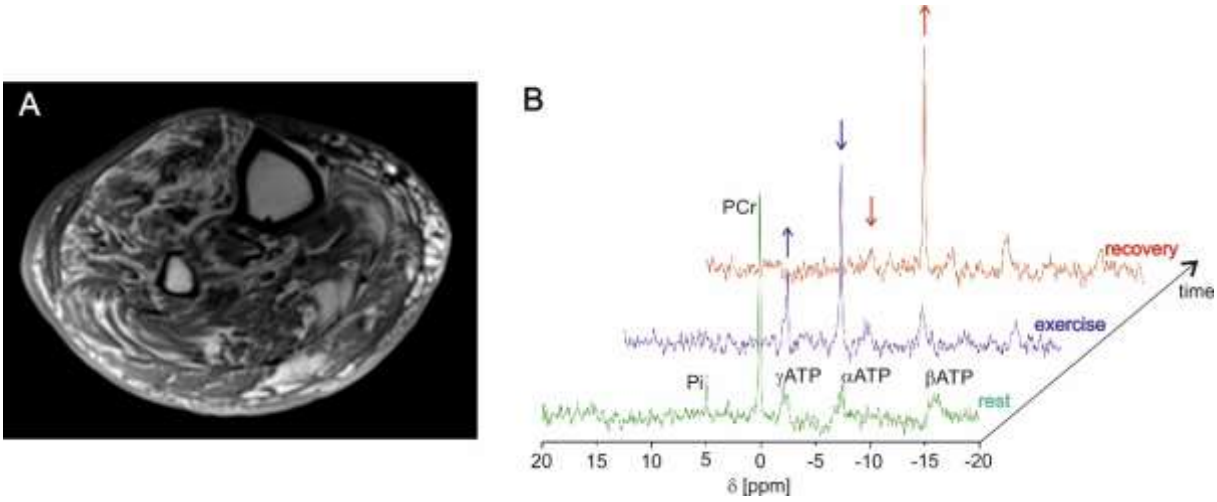


Figure 3. PCr dependency in the rest – exercise – recovery period in one patient before and after ACT.

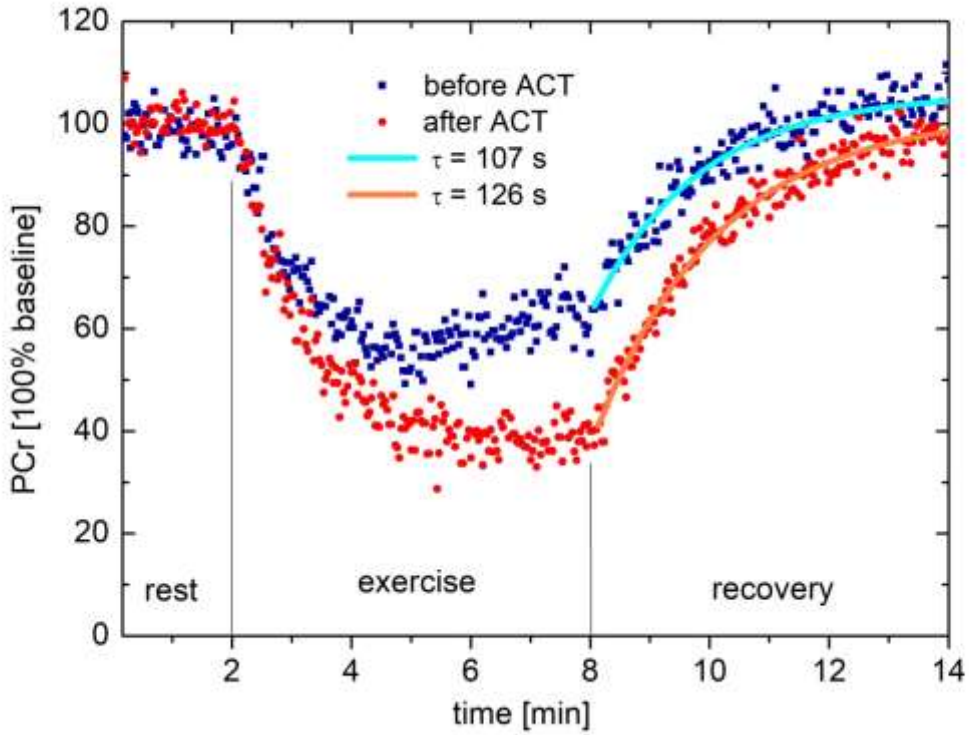
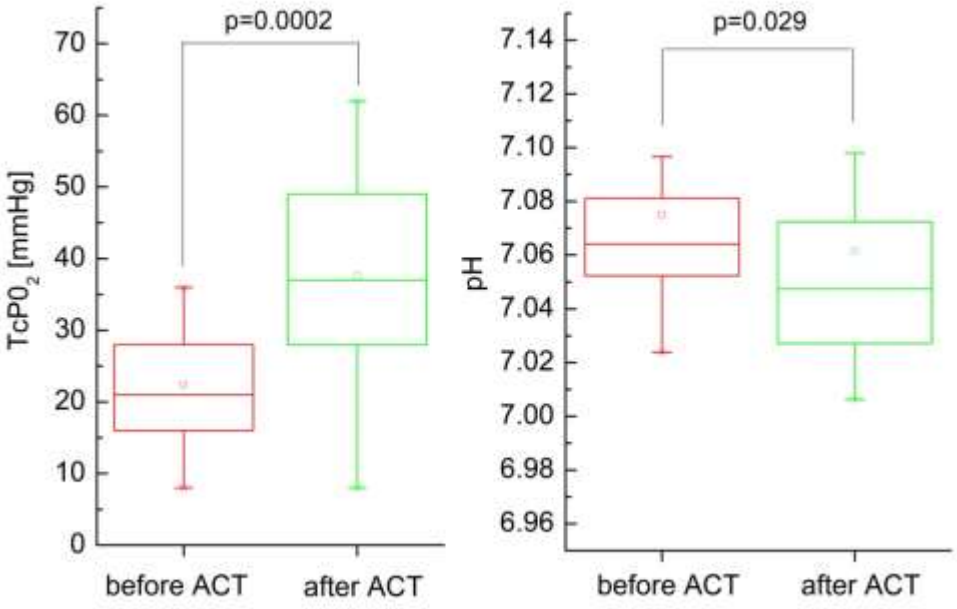


Figure 4. Box plot of $TcPO_2$ and rest pH (MRS data) before and after ACT.



Tables

Table 1. Rest and dynamic parameters of ^{31}P MRS before and after ACT ($TcPO_2$ – transcutaneous oxygen pressure, PCr – phosphocreatine, ATP – adenosine triphosphate, P_{total} – integral sum of phosphorus metabolites, pH_{rest} – rest intramyocellular pH, ΔPCr – drop of PCr during exercise, τ_{PCr} – PCr recovery time, V_{PCr} – initial PCr recovery rate, Q_{max} – mitochondrial capacity, pH_{rest} – intramyocellular pH ~~in~~ at the end of exercise).

	Before ACT	After ACT	P value paired *t*-test/exact sign test*	Standard control values (Sedivy, et al. 2018)
Rest ^{31}P MRS		n=21		
$TcPO_2$	**22.4±8.2**	**37.6±13.3**	**0.0002**	
PCr/Pi	5.1±2.1	5.0±2.0	0.58	8.6±1.6
PCr/P_{total}	0.418±0.080	0.407±0.099	0.66*	0.51±0.03
Pi/P_{total}	0.096±0.043	0.092±0.031	1*	0.060±0.009
ATP/P_{total}	0.073±0.020	0.072±0.022	0.66*	0.088±0.014
pH_{rest}	7.075±0.059	7.062±0.0063	0.029	7.019±0.026
Dynamic ^{31}P MRS		n=12		
$TcPO_2$	**25.3±7.4**	**41±12**	**0.0001**	
ΔPCr [%]	40±15	43±19	0.63	22±11
τ_{PCr} [s]	470±940	610±1230	0.77*	44±19
τ_{PCr} [s] without 3 extreme subjects (τ_{PCr} >200)	122±62	112±46	0.58	
V_{PCr} [mmol/s]	0.12±0.09	0.15±0.11	0.22	0.27±0.17
Q_{max} [mmol/s]	0.24±0.016	0.24±0.019	0.99	0.57±0.24
pH_{exc}	6.87±0.018	6.85±0.015	0.90	7.034±0.036

Significant differences are shown in bold

Table 2. Spearman coefficients of correlation between $\Delta TcPO_2$ and Δ^{31}P MRS parameters (Δ = after – before ACT).

	r	p value
Rest ^{31}P MRS	n=21	
PCr/Pi	-0.20	0.38
ATP/P_{total}	-0.24	0.32
pH_{rest}	0.31	0.18
Dynamic ^{31}P MRS	n=12	
τ_{PCr} [s]	0.15	0.68
Q_{max} [mmol/s]	-0.53	0.078
pH_{exc}	0.05	0.88