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A Pilot Study of Ceramic Powder Far-Infrared Ray Irradiation (cFIR) on Physiology: Observation of Cell Cultures and Amphibian Skeletal Muscle

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Abstract

The purpose of this research was to assess the potential for far-infrared ray irradiation from ceramic powder to improve exercise performance at room temperature. We designed experiments with murine myoblast cells (C2C12) to study the effect of cFIR irradiation on cell viability and lactate dehydrogenase release under H_2O_2-mediated oxidative stress and evaluated intracellular levels of nitric oxide and calmodulin. We also used electro-stimulation of amphibian skeletal muscle. Our results show that cFIR strengthened C2C12 under oxidative stress and delayed onset of fatigue induced by muscle contractions. We discuss possible mechanisms including anti-oxidation and prevention of acid build-up in muscle tissue based, and expect to see more applications of cFIR in the future.

Key Words: cFIR, C2C12, H_2O_2-mediated oxidative stress, LDH release, electro-stimulation, muscle contraction, exercise performance

Introduction

Far infrared rays (FIR) are short electromagnetic waves with wavelengths ranging in the infrared spectrum. Most researchers believe that the optimal wavelengths of FIR, which range from 8 to 14 μm, produce biological effects on humans. Although the exact mechanism by which it works is not fully understood, FIR has been used for many therapeutic purposes including vascular-related diseases and other health-promoting practices.

Previous researches have shown that FIR produces both non-thermal and thermal effects, including the increase of micro-vascular dilation, higher blood flow volume, and slight elevation in regional tissue temperature (5, 13). It is also believed that FIR can promote other intracellular effects at the microscopic level, affecting heat transfer in subcutaneous tissues and other physical-biological processes.

Advanced biological material developed at Taipei Medical University provided cFIR proven to possess high potency at room temperature (10). Our prior biomolecular studies of cFIR demonstrated that it increased nitric oxide (NO) and calmodulin (CaM) and strengthened mammalian immunity while showing antioxidant effects in different cell lines (7-9).

Our previous biomolecular studies of cFIR showed that it had significantly positive effects on intracellular NO and CaM levels in different cell lines such as breast glandular cells, macrophages, fibroblasts and vascular endothelium cells (7, 8). Thus, we attempted the same approach on murine myoblast cells (C2C12) which were obtained *via* selective serial passage of myoblasts cultured from the thigh muscle

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of C3H mice. C2C12 cells have been shown to be capable of cell differentiation (6) like *in vivo* skeletal muscle cells, and this cell line is useful for studying cell differentiation as the cells express the characteristics of muscle proteins and receptors.

In recent years, implications of exercise-induced oxidative stress have been a hot topic in sports physiology. Regular physical exercise has many health benefits including lowered overall mortality and reduced risk of cardiovascular disease, cancer and diabetes. But prolonged exercise and heavy sports training are also associated with chronic injuries and exercise-induced immune impairment including increased susceptibility to infection. Sportsman and their trainers often consume different antioxidant supplements such as vitamin E, ascorbic acid and glutamine (2, 4, 10) hoping to enhance muscle power and speed up recovery from muscle fatigue (11).

In real exercise, repeated activity of skeletal muscle causes a variety of changes in its properties, producing muscular weakening and then fatigue. Human beings who perform prolonged exercise may thus feel sore and weak, and under some pathological conditions, repeated contractions involving stretching can cause muscular degeneration. It is believed that fatigue is caused by the accumulation of lactic acid which produces intracellular acidosis that inhibits myofibril proteins and the contraction mechanism. However, recent studies have led to active debate about whether lactic acid is really the cause of muscle fatigue (12). Nevertheless, studies on rapid onset human muscle fatigue have often shown correlation between the decline of intracellular muscle pH and the reduction of force or power production. Studies have shown that acidification of skeletal muscle fibers reduces isometric muscular contraction force by directly affecting isolated myofibril proteins (1, 3). Regardless of whatever role lactic acid may play, intracellular pH changes can cause contractile dysfunction during fatigue.

In this study, we set up an animal model of amphibian muscle experiment for muscle fatigue, and the purpose was to identify if cFIR exhibited its influence on the contraction of skeletal muscle. In this experiment, we applied electric shock directly to frog gastrocnemius muscle. Prolonged stimulation causes muscle fatigue and weakening of muscle contractions. We recorded continual electro-stimulated contractions of frog gastrocnemius muscle to calculate contraction length, summation load and period to muscle fatigue. This experiment was meant to imitate the pattern of fatigue in human muscle contraction resulting from continual strenuous exercise.

Materials and Methods

FIR Ceramic Powder

The ceramic powder used in this study (obtained from the Department of Radiology, Taipei Medical University, Taipei, Taiwan, ROC) was composed of micro-sized particles produced from several ingredients mainly different elemental components (Figs. 1, a and b). The average emissivity of the ceramic powder was 0.98 at wavelengths of 6~14 μm (determined by a CI SR5000 spectroradiometer), which represents an extremely high ratio of FIR intensity. Many physical, chemical and biological effects can be induced with this ceramic powder at room temperature without direct contact (7, 8, 10).

FIR Sources

Two kinds of cFIR sources were used in the different experiments because we needed different forms to apply on cell cultures and isolated animal tissues (*in vitro* study). To use on cell cultures, 100 g of ceramic powder (cFIR) was enclosed in plastic bags (FIR groups). For use with isolated animal tissue, 10% cFIR powder was mixed with silicon rubber and manufactured as bracelets (YY Rubber Company, Foshan, Guangdong, PRC).

Cell Culture

Determination of cFIR's Effects on C2C12 under Oxidative Stress

C2C12 myoblasts were cultured in DMEM (Gibco, Gland Island, NY, USA) containing 10% FBS (Gibco), 100 U/ml penicillin and 100 μ/ml streptomycin (Gibco) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C for 48 h. After cells reached confluence, the medium was changed by replacing 10% FBS with 10% horse serum (Gibco) to induce differentiation. The medium was replaced every other day during differentiation. After five days, differentiation was complete and experimental procedures commenced. Two groups of cell dishes were divided into a cFIR group (n = 6) and a control group (n = 6). Only the cFIR group was treated with cFIR powder filled in plastic bags and placed beneath the culture dishes, and these were accompanied by the control group for 24 h.

C2C12 Cells Treated with cFIR under H_2O_2-Mediated Oxidative Stress and Assayed for Cell Viability

A cell proliferation kit (XTT) was used as an indicator of cell viability as determined by its mitochondrial-dependent reduction to formazone. Cells were plated at a density of 4×10^5 cells/well into 24-well plates for 24 h, followed by treatment with different concentrations of H_2O_2 (100 μM), then

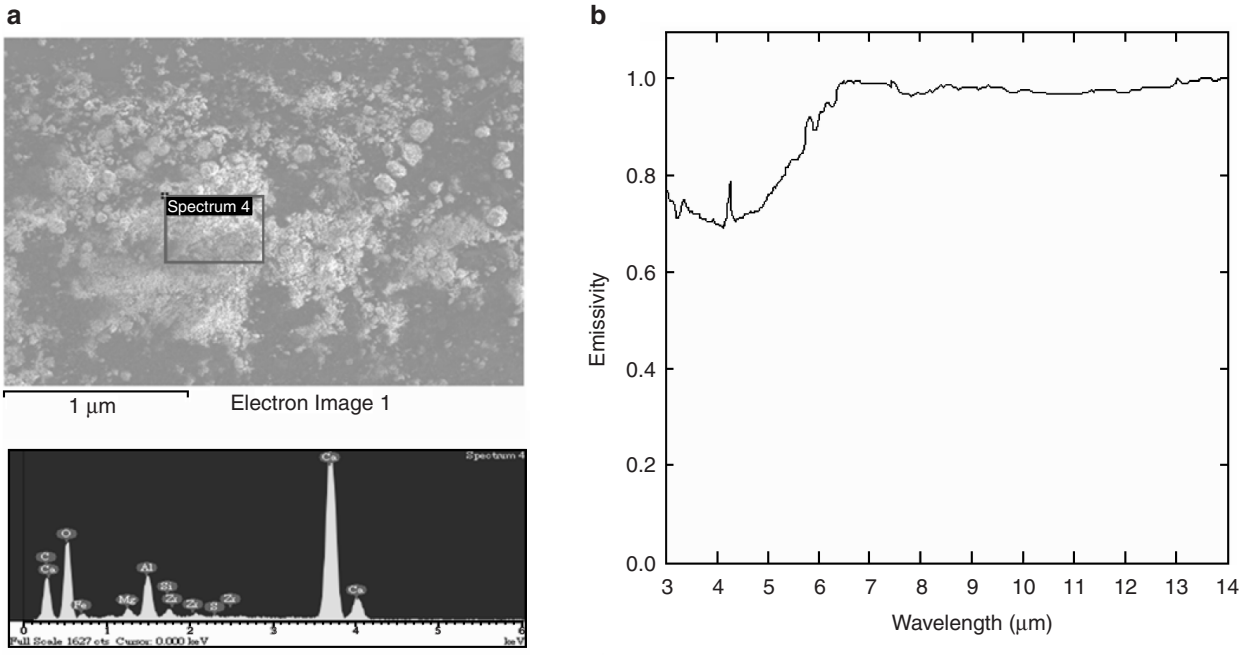

Fig. 1. a. Elemental analysis of the ceramic powder with FIR irradiation using electron microscopy equipment with electron beam processing on the selected spectrum. b. Emissivity spectrum of the FIR ceramic powder determined by a CI SR5000 spectroradiometer (x axis: wavelength [µm] and y axis: emissivity compared with black body radiation source [1.0]).

treated for a further 24 h. Cells were washed three times with phosphate-buffered saline (PBS) (Gibco), and XTT (1 mg/ml) was added to the medium. After three hours, the supernatant was collected. The absorbance was read at 450 nm with an enzyme-linked immunosorbent assay (ELISA) analyzer (Gemini XPS Molecular Devices, Sunnyvale, CA, USA).

Lactate Dehydrogenase (LDH) Activity Release Assay

The percentage of LDH activity release was expressed as the proportion of LDH released into the medium compared to the total amount of LDH present in cells treated with lysis buffer (Roche, Basel, Switzerland). LDH release concentrations in H_2O_2-treated (100 µM) cells were analyzed, and the designated control and cFIR groups were compared. After thirty minutes of incubation, the oxidation activity of NADH at 490 nm was monitored with an LDH assay kit (Roche).

Assay for Intracellular NO by Flow Cytometry

Dishes treated with cFIR powder (the FIR group) and controls were prepared. All dishes were then stained with DAF-FM diacetate for fluorescence measurement. All C2C12 cells were analyzed by a fluorescence-activated cell sorter (FACS) and flow cytometry at the single-cell level. As data were acquired and analyzed, the mean fluorescence intensities of the cells were determined.

Western Blotting to Detect Intracellular Level of CaM Protein

Cells were harvested and washed twice with phosphate-buffered saline (PBS) and centrifuged to remove the PBS. Then a lysis buffer was added, and the mixture was stored at -20°C overnight. The mixture was centrifuged (at 13,000 rpm for 30 min at 4°C), and the supernatant fraction was collected. The protein concentration of the collected supernatant fraction was then assayed. Sample buffer was then added. Samples were prepared in a 100°C water bath and heated for 20 min. For the western blot analysis, proteins were separated, and equal amounts of protein from each sample (50 µg) were carefully loaded. The blots were probed with an anti-CaM antibody (diluted 1:5000, Novus Biologicals, LLC, Littleton, CO, USA) and an anti-β-actin antibody (diluted 1:5000, Sigma, Taufkirchen, Germany). After three washes, blots were probed again with an anti-rabbit immunoglobulin G (IgG) antibody (diluted 1:8000, Sigma) and a rabbit anti-mouse antibody (diluted 1:8000, Sigma) to determine the loading and specificity of the cFIR effect and the control group. The data from individual gels for CaM protein and actin were collected using Winlight32 software (Berthold Technologies, Bad Wildbad, Germany) and subjected to statistical analysis.

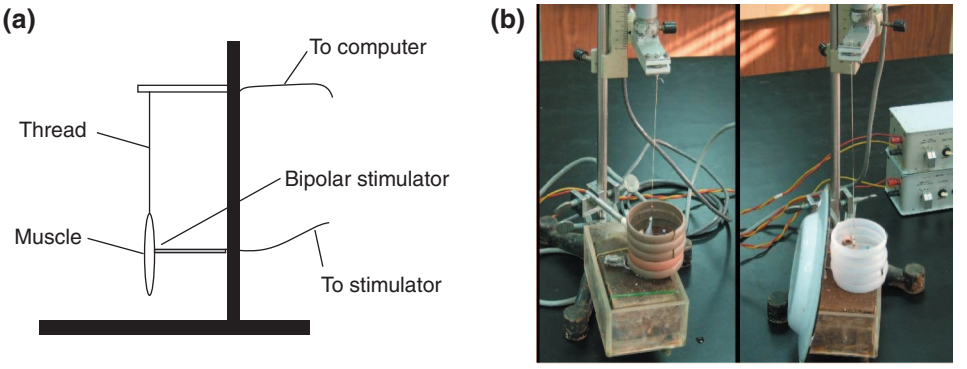

Fig. 2. Schematic view (a) and the actual view (b) of the muscle stimulation experiment. During the electro-stimulation of amphibian skeletal muscles isolate from the same individual frog, the muscles were matched using cFIR bracelets (left, cFIR group) and control bracelets (right, control group).

Isolated Animal Tissue

Observations of cFIR's Effects on Electro-Stimulated Contractions of Amphibian Skeletal Muscle

Firstly, we set up and mounted the gastrocnemius muscle. At the same time, we obtained double-pithed frogs and used a pair of sharp scissors to peel the skin off the leg and trim the thigh muscles away from the femur. Then, we dissected under the Achilles tendon. Finally, we ligated the tendon and freed the gastrocnemius from the tibia and fibula of the lower leg, and cut off the tibia and fibula and femur. The muscle preparation was then attached to the recording apparatus as shown in Fig. 2.

While two individual gastrocnemius muscles were connected to the computer system (BIOPAC physiological recording system, Goleta, CA, USA), we operated the computer and the stimulator to obtain the maximum contraction power by adjusting the power supply. The stimulator stimulated the muscle with continuous pulses at intervals of five ms for periods of one second. The purpose of this experiment was to stimulate the muscle continually for a long period of time, and to record the loading contraction force (grams) and time until onset of muscle fatigue.

Before electro-stimulation of isometric contractions, the right and left side muscles from the same frog were randomized to matching and fully surrounded either by cFIR silicon rubber or ordinary silicon rubber bracelets but without direct contact (Fig. 2).

The contraction force was recorded during continuous electro-stimulation until both muscles were fatigued. Comparison of the mean contraction force (mean time of contraction before complete fatigue) between the cFIR and control groups was performed. A series of experiments with pairs of right and left side gastrocnemius muscles were performed consecutively according to these procedures.

Measurement of pH Changes after Fatigue from Electro-Stimulated Isometric Contractions

Pre-test of the muscles by measuring pH values on the same frog included (a) before electro-stimulation, (b) after 20 min of electro-stimulation of the control muscle and (c) after 20 min of electro-stimulation of the cFIR irradiated muscle.

After the consecutive electro-stimulation of contractions according to the above procedures for the cFIR and control groups, a number of two right and left fatigued muscles in pairs were then frozen in liquid nitrogen. One gram was taken from each of the muscles for homogenization. The muscle samples were homogenized in 5 ml of deionized water and measurement of pH values was subsequently performed. After pH value data were obtained, we compared the ratios between the contraction force and the final pH value of cFIR and the control groups.

Statistical Analysis

Data were expressed as means ± SD. Statistical significant differences between the control and cFIR groups was determined using a paired *t*-test. A value of $P < 0.05$ was considered statistically significant. All statistical analyses were performed with SPSS for Windows 13.0 (SPSS, Inc., Chicago, IL, USA).

Results

Cell Culture

Results of C2C12 Cells Treated with cFIR under H_2O_2-Mediated Oxidative Stress and Cell Viability Assay

According to the XTT assay, cFIR had the ability to prevent toxicity to C2C12 cells under H_2O_2-

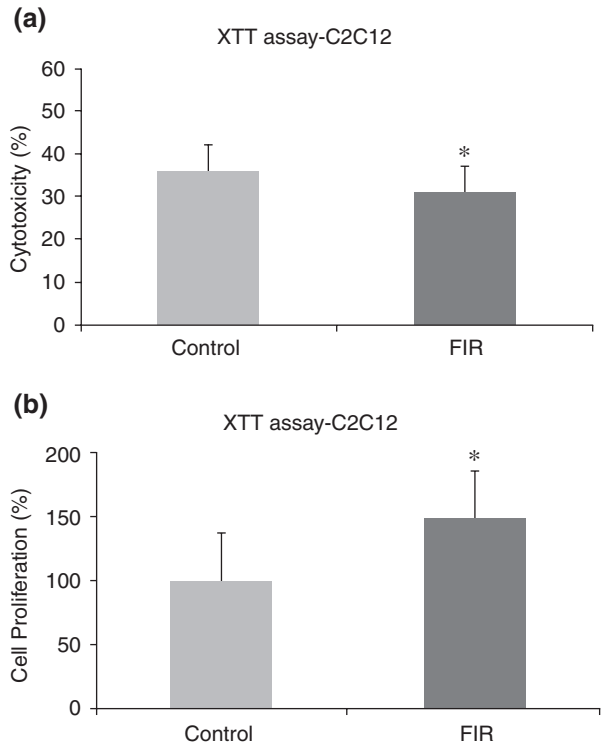

Fig. 3. Box-plots of (a) cytotoxicity (n = 24) and (b) cell proliferation (n = 6) comparison of the two groups under the oxidative stress of 100 μM of H_2O_2. Results show that cFIR possesse the ability to stimulate proliferation of C2C12 cells against hydrogen peroxide (*$P < 0.05$).

mediated oxidative stress (Fig. 3). The percentage of cell proliferation was significantly different in the cFIR and control groups when treated with 100 μM concentrate of H_2O_2 ($P < 0.05$).

Result of LDH Activity Release Assay

Assays to identify the effect of cFIR on LDH release indicated a significant difference between the control and cFIR groups ($P < 0.05$). Results showed significantly reduced LDH release with 100 μM concentrate of H_2O_2 in the cFIR group (Fig. 4).

Measurement of Intracellular CaM Protein Levels

We examined the effect of the cFIR irradiation from ceramic powder on the expression of intracellular CaM protein of C2C12 and the control group after 24 h, and there were no significant differences between these two groups (Fig. 5).

Result of Intracellular NO Levels by Flow Cytometry

Results showed no significant difference in the intracellular levels of NO in the C2C12 cells between

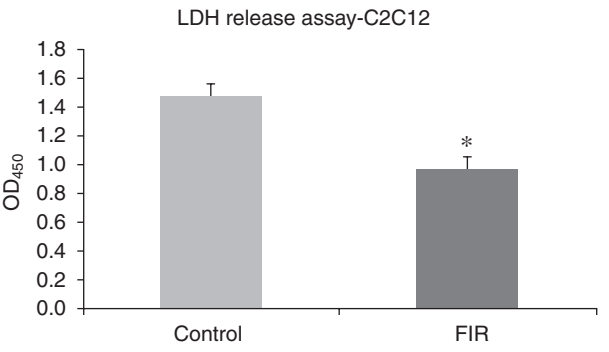

Fig. 4. Box-plot of LDH release assay for C2C12 cells. C2C12 cells treated with 100 μM H_2O_2 showed lower LDH release than control group (n = 8). *$P < 0.05$, significantly different compared with the control group.

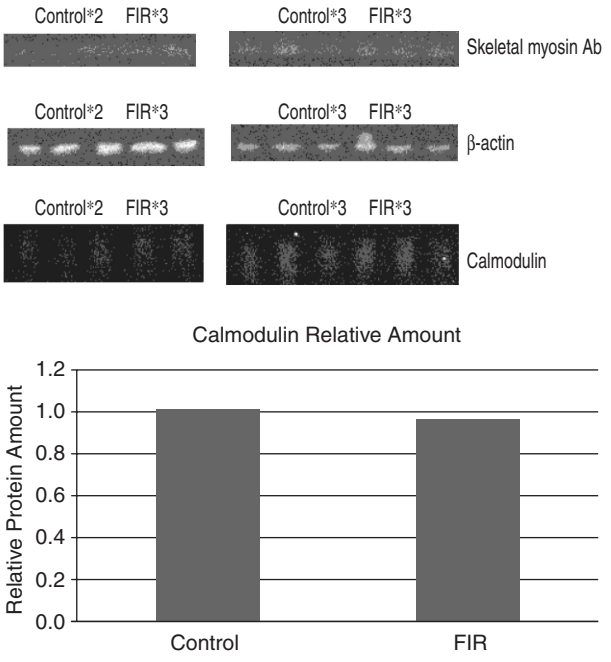

Fig. 5. There was no significant difference in intracellular calmodulin protein level of C2C12 cells with and without cFIR treatment (n = 5) after 24 h.

the cFIR group and the control group (Fig. 6).

Isolated Animal Tissue

Comparison of Electro-Stimulated Contractions of Amphibian Skeletal Muscle for cFIR and Control Groups

In a series of electro-stimulation experiments, though both muscles were from the same amphibian, they showed different contraction forces at the beginning. Even though the initial contractive loading of the cFIR group was weaker than the control group, the cFIR samples continued to contract longer than

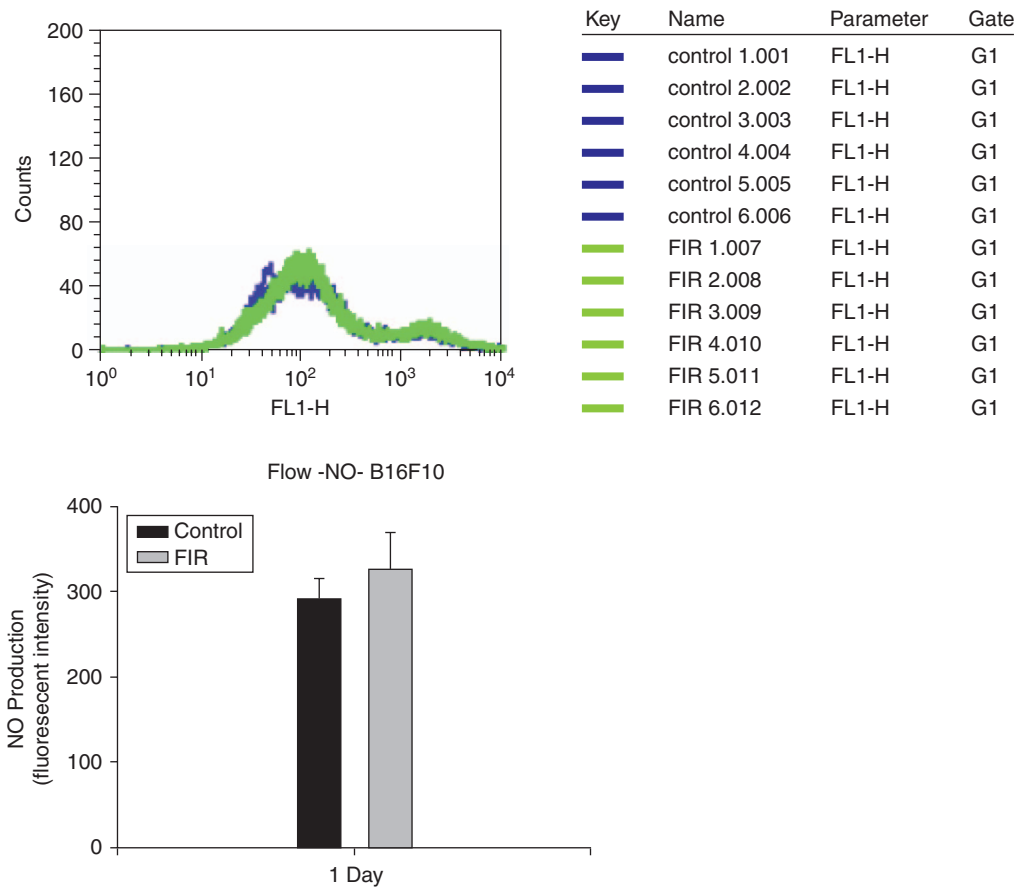

Fig. 6. NO levels of C2C12 with and without cFIR treatment (n = 6) after 24 h as revealed by flow cytometry. It shows a tendency of increase intracellular NO with cFIR irradiation ($0.1 > P > 0.05$).

those of the control group. In addition, there was a significant difference ($P < 0.05$) between the two groups in the mean contraction load (mean duration of contraction before complete fatigue). Results indicated that cFIR bracelets could prevent fatigue in amphibian muscles (Fig. 7).

pH Changes after Fatigue by Electro-Stimulated Isometric Contractions

Fig. 8 shows (a, left bar), the initial pH value of the frog muscle before electro-stimulation, (b, middle bar), after electro-stimulation of the control muscle after 20 minutes of electro-stimulation of the control muscle and (c, right bar), after 20 min of electro-stimuation of cFIR-irradiated muscles. This experiment showed that metabolic acid accumulation after electro-stimulation caused a measurable pH value decrease in muscles, and the presence of cFIR could normalize acidification following muscle contractions.

We further analyzed the differences in pH and total contractions ($P < 0.01$) between these two groups after the above electro-stimulation experiment (Table 1).

Discussion

In this study, we found a tendency for intracellular nitric oxide to increase in C2C12 cells after continuous irradiation with cFIR for 24 h ($0.1 > P > 0.05$). However, we failed to obtain significant results on CaM proteins in contrast to our previous studies showing that the cells could respond to irradiation by cFIR. The most important and conclusive results show that cFIR has an antioxidant effect on these cells, based on cell viability percentages and LDH release assays.

In recent years, the implications of exercise-induced oxidative stress have been a hot topic in sports physiology. It is clear that continually contracting skeletal muscle generates free radicals, and prolonged intense exercise will, therefore, result in oxidative damage. Although low levels of oxidants may play multiple regulatory roles in producing skeletal muscle force, many studies are investigating exercise and free radical production by focusing on the damaging effects of oxidants in the muscle. It is certain that high levels of free radicals can damage cellular components. Nonetheless, high levels of

Table 1. pH values and total contractions after fatigued by electro-stimulation isometric contractions

	Control (n = 10)	FIR (n = 10)	*P*
pH	6.22 ± 0.17	6.29 ± 0.14	0.0671
Total Contraction	19.07 ± 13.93	26.58 ± 17.28	0.0020**

**$P < 0.01$, significantly different compared with the control group.

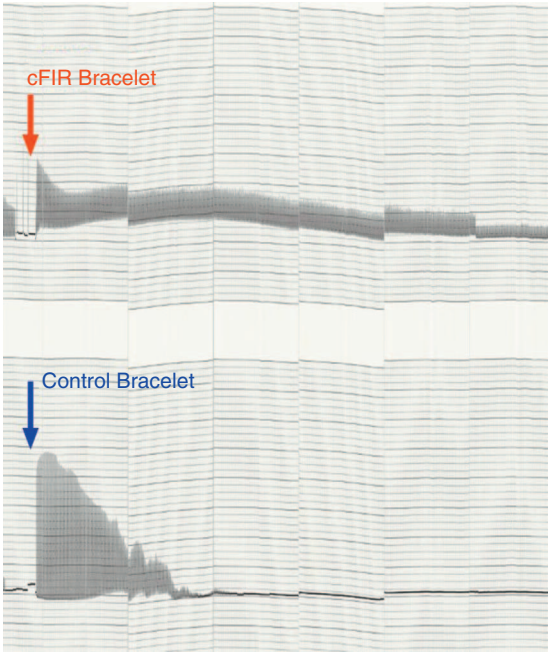

Fig. 7. Gastrocnemius muscle contraction record charts. cFIR group had prolonged the fatigue process of the muscle, even though the initial contraction force was weaker than the control group.

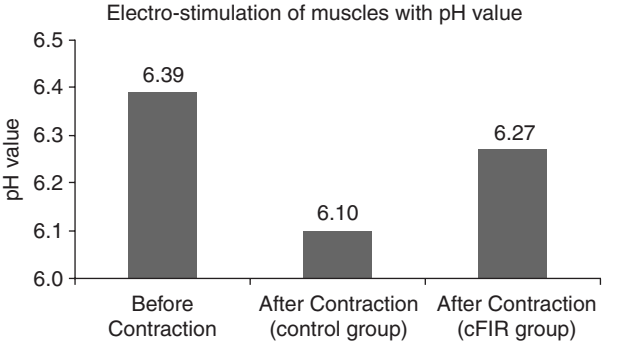

Fig. 8. Box-plot of pH value for the frog gastrocnemius muscle samples. After long-term isometric contraction by electro-stimulation, the pH change of the FIR group (0.12) was smaller than the control group (0.29).

ROS (free radicals of oxidants) promote skeletal muscle contractile dysfunction resulting in muscle fatigue.

On the other hand, in recent years, since the implications of exercise-induced oxidative stress has been a hot topic in sports physiology, preventing post-exercise oxidative stress through antioxidant intervention seems to be a reasonable approach to improving sports performance. Hence, muscle contraction models had been carried out (2, 4, 10, 11). This experiment was designed to imitate human muscle contraction and the pattern of fatigue resulting from continuous strenuous exercise.

In this pilot studies of cFIR's effects on cell cultures (murine myoblasts) and amphibian skeletal muscles, we have found that cFIR may reduce muscular fatigue and normalize acidification of contracted muscles. The beneficial effects of cFIR for delaying onset of fatigue might have originated from its anti-oxidant properties and preventing metabolic acidosis of muscular fiber. We hope that convenient, non-invasive and external application of this kind of ceramic material will receive further attention.

Acknowledgments

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