Diagnosis

Effects of extracorporeal pulse activation on *in-vitro* lipopolysaccharides-treated chondrocytes

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Abstract

Introduction
Osteochondritis dissecans is a condition where a segment of articular cartilage with its underlying subchondral bone gradually separates from the surrounding osteocartilaginous tissue. The extracorporeal pulse activation treatment has been used to treat various tendinopathies such as calcific tendonitis of the shoulder, lateral epicondylitis and plantar fasciitis. It is also used with success in delayed fracture unions and non-unions. However, extracorporeal pulse activation effect on cartilaginous tissue (i.e. osteochondritis dissecans lesion) is unclear. Efficacy and mechanism of the extracorporeal pulse activation on the *in-vitro* chondrocytes have not been reported. Our current study is to investigate how different dosages of the extracorporeal pulse activation treatment affect cell viability and changes of cell morphology, oxygen consumption, nitric oxide as well as cytokine interleukin-1β release in lipopolysaccharides-induced inflammatory chondrocytes.

Materials and Methods
Human chondrocyte cell line was maintained in an incubator at 37°C and 5% CO₂. Cells were divided into four groups with different extracorporeal pulse activation treatments:

1. Control
2. Low dose (0.1 mJ/mm², 6.0 Hz and 2000 impulse)
3. Middle dose (0.25 mJ/mm² and 4.0 Hz)
4. High dose (0.55 mJ/mm² and 3.0 Hz)

A series of analyses was included: mitochondrial function measurement, measurements of O₂ uptake and nitric oxide release, cell viability and enzyme-linked immunoabsorbant assay. The middle dose EPA demonstrated more reduction in oxygen uptake and IL-1β release than those in the low dose EPA.

Discussion
The middle dose extracorporeal pulse activation is able to attenuate lipopolysaccharides-induced chondrocyte inflammation and apoptosis with increases of cell viability. The mechanism of extracorporeal pulse activation may relate to the restoration of mitochondrial function and regulation of nitric oxide and cytokine release.

Conclusion
Extracorporeal pulse activation may provide a new approach to treating chondral defects such as osteochondritis dissecans and inflammatory joint conditions.

Introduction
Osteochondritis dissecans (OCD) is a condition where a segment of articular cartilage with its underlying subchondral bone gradually separates from the surrounding osteocartilaginous tissue. Surgery is often the best option in the treatment of unstable OCD. Surgical results are best if done before the OCD affected area becomes loose or detached. According to Wright et al., only 35% of patients have a good or excellent result after excision of a partially detached or loose fragment based on a 4–15 year follow up. Linden found that 38 of 48 patients had osteoarthritis of the knee on an average 33-year follow up. With stable OCD lesions in juvenile patients non-operative treatment has good success depending on age. This treatment may include bracing and non-weight bearing, which often involves very little joint movement and can result in joint stiffness as well as atrophy of the quadriceps. Recently extracorporeal shock wave (ESW) has been used to treat various tendinopathies such as calcific tendonitis of the shoulder, medial and lateral epicondylitis of the elbow and plantar fasciitis. It is also used with success in delayed fracture unions and non-unions. However, effect of ESW on cartilaginous tissue is unknown. Extracorporeal pulse activation treatment (EPAT) has been used successfully to treat the OCD lesion. There is a need to understand the mechanism of the ESW on the *in-vitro* chondrocytes.

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Mitochondria are complex organelles that oxidise a wide range of metabolic intermediates. Adenosine triphosphate (ATP) is generated by the activity of an electrogentic proton pump that spans the inner mitochondrial membrane. Mitochondrial impairment and defective oxidative phosphorylation have been involved in cell apoptosis which linked to a variety of human disorders. The analysis of mitochondrial respiratory chain (MRC) activity in the degenerative chondrocytes showed a significant decrease in complexes II and III in comparison with normal chondrocytes. Nitric oxide (NO) is a messenger implicated in both chondrocyte death and protection from oxidative damage induced on chondrocytes. A variety of NO donors have been shown to suppress energy production by mitochondrial respiration in different cell types. Therefore, our current study was to investigate how different dosages of extracorporeal pulse activation (EPA) treatment affect cell viability and changes of cell morphology, oxygen consumption, NO as well as cytokine interleukin (IL)-6 release in lipopolysaccharides (LPS)-induced inflammatory chondrocyte.

Materials and Methods

The protocol of this study has been approved by the relevant ethical committee related to our institution in which it was performed.

Cell culture

A chondrocyte cell line (CHON-001) was purchased from American Type Cell Culture (Manassas, VA). The cell culture was maintained in an incubator at 37°C and 5% CO2. Briefly, the following subculture procedures were performed: the subculture was allowed to reach approximately 80% confluence in 75 cm² flasks. The spent media was removed and discarded. The cell layer was rinsed with 0.25% (weight/volume) trypsin–0.53 mM ethylenediaminetetraacetic acid (EDTA) solution to remove any traces of residual bovine calf serum, and 0.25 mL of Trypsin–EDTA solution was added to the flasks, which were placed into an incubator for approximately 10 min. Five millilitres of Dulbecco’s Modified Eagle’s Medium was added to the cultures to inactivate the trypsin and the cell numbers in each group are made equal.

Protocols

The cells were divided into four groups with control, low, middle and high dose of EPA. The LPS group was given 200 μg/mL after EPA treatment. Each EPA-treated group received a low (0.1 μl/mm², 6.0 Hz and 2000 impulse), middle (0.25 μl/mm², 4.0 Hz and 2000 impulse) and high (0.55 μl/mm², 3.0 Hz and 2000 impulse) dosage of EPA, respectively.

Extracorporeal pulse activation treatment

EPAT system (Duolith SD1®, Storz Medical AG, Postfach, Switzerland) combines several proprietary technologies, including high-energy focused cylindrical-source electromagnetic (F-SW) technology that we used in this study. It has a short pulse length and is concentrated on areas of a few millimetres in diameter. We used an F-SW handpiece with standoff device I, which is able to provide a therapeutically effective penetration depth up to 105 mm. Its focal zone is 30 mm in diameter and its depth of focal zone ranges from 15 to 45 mm. An effective distance from the surface of handpiece to the centre of focal zone is approximately 30 mm. Cells were transferred to aseptic 2.0 mL vials for EPA treatment. A shock wave generator produced shock waves and directed them to the vial of cultured cells. The F-SW handpiece was placed under a specifically designed holder in which the vial with the cells sit on the centre point of the focal zone and the distance between the F-SW emitting surface and vial is 10 mm. Each vial received one administration of 2000 shots at their prescribed dosage during the entire experiment. After EPA therapy, the chondrocytes were cultured in 24-well culture plates with LPS 200 μg/mL treated under no serum medium at a density of 1.3 × 105.

Mitochondria isolation

The mitochondria were isolated cells/well. Twenty four hours after plating, the cells were harvested for morphologic analysis (apoptosis), cell viability and mitochondrial function assay (n = 6 for each group). Using Pierce mitochondria isolation kit (Thermo Fisher Scientific, Rockford, IL) and following the manufacturer’s protocol. Briefly, 2 × 107 cells were pelleted by centrifugation at 850g for 2 min, and Mitochondria Isolation Reagent A was added to the pellet. Cells were vortexed for 5 s and then incubated on ice for 2 min. Mitochondria Isolation Reagent B was added and then followed by vortexing for 5 s. Tubes were incubated on ice for 5 min and vortexing was being done every 1 min. Mitochondria Isolation Reagent C was added, and the tubes were inverted several times to mix. The supernatant obtained by centrifugation at 700g for 10 min at 4°C was transferred to a new tube and centrifuged at 3000g for 15 min. Mitochondria Isolation Reagent C was added to the pellet and centrifuged at 12,000g for 5 min. The pellet contains the mitochondrial fraction.

O2 uptake and nitric oxide release

The determination for O2 uptake (consumption) and the levels of NO release were measured polarographically at 28°C using a computer controlled with an oxygen electrode and an NO electrode (World Precision Instruments, Sarasota, FL) concomitantly. Calibration of O2 uptake and NO release was performed...
according to the manufacturer’s instructions by using aqueous calibration for oxygen sensors and NaNO₂ and KI+H₂SO₄ for NO. The calibration curve exhibited a linear correlation coefficient of 0.98 and 0.99.

**Cell viability assay**

Cells were seeded at **1 × 10⁴** cells/well in 96-well plates. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; 5 mg/mL; Sigma, St Louis, MO, USA) was added to each well and incubated for **4 h** at **37°C**. The formazan product was dissolved by adding **150 μL** dimethyl sulfoxide to each well. The MTT absorbance value was detected at **490 nm** by the microplate reader (Modulus Microplate, Turner Biosystems, Sunnyvale, CA, USA).

**Enzyme-linked immunosorbant assay**

The cells were in ice cold lysis buffer and used for enzyme-linked immunosorbant assay (ELISA) and protein measurements. Levels of IL-1β in cells with or without LPS-treated were determined by murine-specific Duo Set ELISA development kits (R&D Systems, Minneapolis, MN). Data are expressed as a function of protein (pg/mL).

**Statistics**

Data analyses were performed with SPSS, version **10.0** (SPSS, Chicago, IL). Data were reported as the mean ± SD. Comparisons between groups were carried out using one-way analysis of variance. Test was used to determine changes to the differences among the control, low, middle, and high dose of EPA groups in a normal distribution. A value of **P < 0.05** was considered statistically significant.

**Results**

**Effects of lipopolysaccharides on chondrocytes morphology**

After incubation of **200 μM/mL** LPS chondrocytes presented with apparent apoptosis in larger areas: an irregular chondrocyte shape, absent membrane, absent nucleus and cell fragmentation (Figure 1). Following the EPA treatment, both low and middle dose groups displayed significant improvements of cell viability, estimating one-third of cells being normal ellipsoid appearance and normal colour in the low dose group, while half of cells have normal morphology in the middle dose group. However, cells morphology deteriorated and apoptosis worsened in the high dose group as compared with the control group. Overall the highest value of cell viability was recorded in the middle dose group.

**Effects of extracorporeal pulse activation on the cell viability of chondrocytes**

Figure 2 shows that middle dose of EPA significantly increased the cell viability of chondrocytes (**3.79 ± 0.1, P < 0.05**) compared with control (**3.26 ± 0.17**), low dose (**3.38 ± 0.09**) and high dose (**2.87 ± 0.06**). Consistent with morphology result, the high dose EPA group significantly reduced value in the cell viability than control subgroup (**P < 0.05**).

**Effects of extracorporeal pulse activation on mitochondrial oxygen uptake**

Oxygen uptake on mitochondrial function of the chondrocytes was successfully detected, where all EPA groups significantly reduced oxygen consumption (Figure 3). The middle (**11.9 ± 1.1**) and high dose EPA groups (**11.3 ± 1.1**) decreased the oxygen uptake the most compared with

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Effects of extracorporeal shock wave on interleukin-1β expression

In Figure 5, IL-1β expression in low (25.1 ± 3.9) and middle dose of EPA group (23.5 ± 3.8) significantly decreased compared with control group (34.2 ± 3.9, \( P < 0.05 \)). There is no difference between control group and high dose of EPA (29.1 ± 3.6).

The LPS incubation and nutrition-deprived condition-induced chondrocytes are prone to EPA treatments and their biologic and biochemistry responses were dosage dependent. Morphologically, both the low and middle dose EPA subgroup resulted in improvement, including reduced zone of cell degeneration and apoptosis as well as reduction of cell swelling, whereas the high dose subgroup increased cell inflammation and apoptosis. The viability of chondrocytes was consistent with morphological changes of the middle dose.

Discussion

The middle dose EPA exhibited the highest value of the cell viability and the high dose EPA had the lowest value of the cell viability. Additionally, the middle dose EPA demonstrated more reduction in oxygen uptake and IL-1β release than those in the low dose EPA. However, the higher dose EPA produced no changes of NO release and IL-1β release as compared with the control group. These data further imply that the middle dose EPA is an appropriate dose to improving pathological chondrocytes.

A limited number of studies for the dose-related effects of ESW in orthopaedic applications were reported.
dose-related histological changes with the application of ESW to the Achilles tendons of the rabbits have been investigated\textsuperscript{22}. They found a marked infiltration of inflammatory cells, fibrinoid necrosis and fibrosis of the paratenon as severe histological alterations were observed with the application of 1000 impulses of 0.60 ml/mm\textsuperscript{2}. They concluded that shock waves with energy-flux densities over 0.28 ml/mm\textsuperscript{2} should not be selected in clinical trials. In another study, they used 0.28 ml/mm\textsuperscript{2} as the upper limit of ESW in over 300 patients with no harm as assessed by ultrasound or magnetic resonance imaging. In the current study, we also demonstrated a dose-related effect of EPA on cell viability and activity of mitochondrial electron respiratory chain. Our results showed that the middle dose (0.25 ml/mm\textsuperscript{2}) significantly improved the cell viability and reduced cell apoptosis in nutrition-deprived and LPS-treated chondrocytes. In contrast, the higher dose (0.55 ml/mm\textsuperscript{2}) decreased cell viability and increased cell inflammation and apoptosis. Therefore, we recommend 0.25 ml/mm\textsuperscript{2} as an effective dose in the treatment of children with OCD.

Chondrocyte matrix synthesis and mineralisation are modulated by the balance between ATP generation and consumption. Blanco et al.\textsuperscript{23} suggested that the chondrocyte mitochondria are specialised for calcium transport and are important in the calcification of the extracellular matrix. The analysis of MRC activity in osteoarthritic human articular chondrocytes showed a significant decrease in complexes II and III compared with normal chondrocytes\textsuperscript{24}. While our study in-vitro chondrocyte with LPS and no serum incubation demonstrated higher oxygen uptake, and these oxygen consumptions were significantly decreased by all three different dose EPA treatment, especially by the middle and high dose EPA. These results indicated

**Figure 4:** Comparison of NO release with and without different doses of EPA treatment: means ± SD.

\textsuperscript{**}P < 0.01 compared with control group \((n = 6)\).

**Figure 5:** Comparison of IL-1\textbeta release with and without different doses of EPA treatment: means ± SD.

\*P < 0.05 compared with control group \((n = 6)\).

Orhan et al.\textsuperscript{21} observed the effects of ESW at different intensity applications on the Achilles tendons of the rat and showed that ESW application at high intensity is associated with detrimental tissue effects. Additionally, the extent of tissue injury caused by ESW is dose related. The
that EPA may attenuate the MRC activity and trigger the anti-inflammatory action, which further reduces the inflammation-induced oxidative stress and restore the mitochondrial normal function.

NO is a multifunctional molecule that mediates various biologic processes, including relaxation of smooth muscles, host defense mechanisms against pathogens and inflammation\(^2\). Massive amounts of NO produced by inducible nitrous oxide synthase (iNOS) under pathological conditions (e.g. inflammatory diseases) are potentially harmful, especially when time-spatial regulation of iNOS expression compromise. As a consequence of up-regulation of chondrocyte inducible NO synthase induced by IL-1 and other factors\(^3,26,27\), increased amounts of nitric oxide caused the pathogenesis of the joint. Ciampa et al.\(^28\) assumed that in early stage of inflammation with normal nutrition, ESW inhibit the up-regulation of iNOS in chondrocytes induced by cytokine factors. In late stage of inflammation in which little nutrition condition, ESW may exert a counteracting effect on the cytokine-induced drop in constitutive NOS because of late stage activity\(^29\). Our results implies that the middle dose EPA significantly reduced IL-1β production that triggers the up-regulation of iNOS in the LPS-treated and nutrition-deprived chondrocytes rather than the down-regulation of iNOS. It suggests that the EPA has different effects on synthesis of NO at different stage of inflammation and may regulate proper amounts of NO for a role of anti-inflammatory action.

**Conclusion**

The EPA with optimal middle dose of 0.25 mJ/mm\(^2\) is able to attenuate LPS-induced inflammation, cell death and increase the cell viability in our chondrocyte model. The mechanism of the EPA may relate to the improvement of mitochondrial function and regulation of NO and IL-1β release. The high dose EPA demonstrates more detrimental effect on the inflammatory chondrocytes. The EPA may provide a new approach to treating chondral defects such as OCD and inflammatory joint conditions.

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**Abbreviations list**

ATP, adenosine triphosphate; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; EPA extracorporeal pulse activation; EPAT, extracorporeal pulse activation treatment; ESW, extracorporeal shock wave; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharides; MRC, mitochondrial respiratory chain; NO, nitric oxide; NOS, nitrous oxide system; OCD, osteochondritis dissecans.

**References**

Research study