Lasers in Surgery and Medicine

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Journal:	*Lasers in Surgery & Medicine*
Manuscript ID:	LSM-14-0071.R1
Wiley - Manuscript type:	Basic Science
Date Submitted by the Author:	n/a
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Key Words:	ex vivo, laser therapy, proteomics, synovial membrane

Effects of laser treatment on the expression of cytosolic proteins in the synovium of patients with osteoarthritis

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Grant: This work was supported by grant No 097/2009 from the Medical Research Council (ETT) of Hungary.

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Abstract

Background and Objective: Low level laser therapy (LLLT) has been developed for non-invasive treatment of joint diseases. We have previously shown that LLLT influenced synovial protein expression in rheumatoid arthritis. The aim of this study was to assess the effects of laser irradiation on osteoarthritic (OA) synovial protein expression.

Study Design/Materials and methods: The synovial membrane samples removed from the knees of 6 OA patients were irradiated *ex vivo* using near infrared diode laser (807-811 nm; 25 J/cm^2). An untreated sample taken from the same patient served as control. Synovial protein separation and identification were performed by two-dimensional differential gel electrophoresis and mass spectrometry, respectively.

Results: Eleven proteins showing altered expression due to laser irradiation were identified. There were three patients whose tissue samples demonstrated a significant increase ($p<0.05$) in mitochondrial heat shock 60kD protein 1 variant 1. The expression of the other proteins (calpain small subunit 1, tubulin alpha-1C and beta 2, vimentin variant 3, annexin A1, annexin A5, cofilin 1, transgelin and collagen type VI alpha 2 chain precursor) significantly decreased ($p<0.05$) compared to the control samples.

Conclusions: A single diode laser irradiation of the synovial samples of patients with osteoarthritis can statistically significantly alter the expression of some proteins *in vitro*. These findings provide some more evidence for biological efficacy of LLLT treatment, used for osteoarthritis.

Key words: ex vivo, laser therapy, proteomics, synovial membrane

Introduction

Studies of the expression of proteins (proteomics) play a more and more important role in the investigation of various clinical entities (1). This technique has been successfully used in studying the expression of cytosolic proteins of the synovial membranes in rheumatoid arthritis (RA), osteoarthritis (OA) and ankylosing spondylitis (AS). Differences were found in the protein expression of calgranulin A, vimentin, α-enolase, fructose biphosphate aldolase A in the synovial membrane of patients with RA and AS compared to OA (2). The identification of new biological and clinical markers of inflammatory rheumatic diseases (RA, AS) opened new possibilities of further dissecting the synovial effects of low level laser therapy (LLLT) (3). Bjordal et al. (4) proved the anti-inflammatory and pain-relieving effects of LLLT in the infrared wavelength range in Achilles tendinitis in a randomized, placebo controlled study. Several authors have reported the beneficial clinical results of LLLT in OA and tendinopathy (5). In *in vitro* studies LLLT decreased inflammation by diminishing the production of PGE2 and by inhibiting cyclo-oxigenase 2 (6-13). Rizzi et al. (14) showed that LLLT reduced the inflammatory response induced by trauma in animals by blocking the effects of reactive oxygen species (ROS) and the activation of nuclear factor kappa B (NFκB). Bo et al. (15) also suggested the importance of proteomics in understanding disease pathogenesis in arthritides and in identifying new therapeutic targets. Finally, we have recently showed that the expression of some proteins in the rheumatoid synovial membrane significantly changed following laser irradiation (16).

In the present study we wished to determine whether the expression pattern of synovial proteins in OA would be affected by *ex vivo* laser irradiation.

Patients and methods

Patients

Synovial samples of six patients with knee OA, according to American College of Rheumatology (ACR) classification criteria, were studied. The clinical and laboratory characteristics of our patients are shown in Table 1.

Sample preparation and protein extraction

Following surgery, OA synovial membrane samples were immediately placed into Medium 199 (Sigma Aldrich) solution at 4 ºC. First the fatty and connective tissues were removed, then two 0.5 cm^2 pieces from the macroscopically identical, adjacent parts of synovial membranes were excised. For the *in vitro* irradiation of synovial membrane samples, an approximate mean radiant exposure of 25 J/cm^2 was chosen to model real exposure during clinical treatment (16). Half of the samples were irradiated by laser at distance of 8 cm from the aperture in Petri dishes containing M199 medium. The irradiated area was about 1 cm^2.The control samples were kept under identical conditions, but were not irradiated. Laser irradiation was carried out using a KLS-500 type diode laser (Laseuropa Ltd, Budapest, Hungary), a laser instrument similar to ones used in clinical practice of the authors. The parameters of the laser were as follows: wavelength: 807-811 nm; power: 448 mW; output aperture size: 2 mm x 4.5 mm; beam divergence: 5º. Following irradiation, the samples were incubated at 37 ºC for 21 hours in M199 medium in a 5 % CO_2 thermostat (Heto-Holten). The samples were stored at -70 ºC until further use. In order to extract proteins, the frozen samples were homogenized using a homogenizer (Janke&Kunkel Ika-Werk Ultra Turrax) on ice in 300-300 µl lysing buffer (7 M urea, 2 M thiourea, 30 mM Tris, 4 % CHAPS, pH 8.5). The solution underwent ultrasound treatment for 1 minute (Bandelin Sonorex TK52) then it was centrifuged (12,000 g, one hour, 4 ºC). The proteins in the supernatant were precipitated by acetone (1:4 v/v), then they were centrifuged (3,000 g, 10 minutes, 4 ºC). The protein samples were stored in a lysing buffer at -20 ºC until analytical processing.

Separation of proteins by two-dimensional differential gel electrophoresis (2D-DIGE)

The pH of protein samples was set at 8.0 and then the protein concentration of the samples was determined using PlusOne Quant Kit (GE Healthcare Hungary, Budapest) according to manufacturer's protocol. From each sample, 5 µg of proteins were labelled with CyDye DIGE Fluor Labelling kit for Scarce Samples (GE Healthcare) dye (4 nmol of dye / 5 µg of proteins) according to protocol. Samples containing 2.5 µg of proteins from each control and each laser-treated sample got into the pooled (internal standard) sample. This pooled sample was labelled by Cy3, whereas the control and the treated samples were labelled by Cy5. Then the two differently labelled samples (5 µg of samples and 5 µg of pooled samples labelled by Cy5 and Cy3, respectively) were mixed. Next, 2 mg/ml ditio-treitol (DTT) and 5 µl/ml of IPG buffer were added fresh to our buffer (8 M of urea, 4 v/v% of CHAPS, 15 v/v%

of glycerine), the mixed samples were completed to 450 µl. The samples were then rehydrated on 24 cm IPG strips (pH 3-10 NL, GE Healthcare) for 14 hours at room temperature. Rehydration was followed by the isoelectric focusing of the proteins for 23.5 hours using Multiphore II (GE Healthcare) equipment. The settings were the same as reported previously (16). After this the focused proteins were reduced with / in an equilibrated solution for 20 minutes (1.5 M of Tris, 6 M of urea, 30 % of glycerine, 20 % of sodium dodecyl sulphate (SDS), 0.1 % of bromide-phenol blue, 1 µl/ml of mercaptoethanol), then the strips were placed on SDS polyacrylamid gels of 10 % and were fitted with agar solution of 0.5 %. The gels were run in running tubs filled up with running buffers (Ettan Dalt Six, GE Healthcare) with of the following parameters: 60 minutes, 18 ºC, 2 W/gel; then: 6 hours, 18 ºC, 12 W/gel. At the end of running the gels were scanned by Typhoon TRIO + device at a wavelength corresponding with the applied CyDye dyes. The scanned pictures were evaluated with the DIA (DeCyder Differential Image Analysis) and BVA (DeCyder Biological Variation Analysis) programs. The protein spots were identified in which the amount of proteins were significantly different ($p<0.05$) in the treated sample compared to the control.

Protein identification using preparative gel and data analysis

Protein samples of 800 µg were added to the preparative gel. During the first dimensional run the protocol corresponding to the analytical gel was applied. Deviation occurred just at the second dimensional run. The glass sheets used for preparing the gel had been treated with Blind Silane solution (ethanol: 80 %, ice vinegar acid: 0.02 %, blind silane: 0.001 %, ddw: 18 %). After the run, the gels were fixed for an hour (20 % of methanol, 1 % of phosphoric acid), then they were dyed using methanol:A:B=2:8:1 solution. (Solution A: 0.3 M of ammonium-sulphate, 2.4 % of phosphoric acid; solution B: 5 % of Coomassie Blue G-250) for one week. Then gels were neutralized using Tris-solution of 0.05 M (pH 6.5) for 3 minutes, then it was washed with methanol of 25 %, finally it was stabilized by ammonium-sulphate of 0.75 M for 8 hours. Based on the results of the analytical gel, the identical protein spots of the preparative gel were excised. These were evaluated in the Department of Chemistry at University of Szeged by liquid chromatography-mass spectrometry (LC-MS/MS) analysis. For the identification of proteins the following criteria were used: protein score > 66 (see Table 2) and level of significance of $p<0.05$ were considered as significant.

Results

Altogether 11 proteins exerted differential expression in LLLT reated OA synovial tissue samples as compared to untreated control samples (Table 2).

Mitochondrial heat-shock 60kD protein 1 variant 1 (mhsp60) was the only protein showing significantly increased expression in synovial tissue of three patients following LLLT compared to untreated synovial tissue ($p<0.05$).

Other synovial proteins, such as calpain small subunit 1, tubulin alpha-1C and beta 2, vimentin variant 3, annexin A1, annexin A5, cofilin 1, transgelin and collagen type VI alpha 2 chain precursor exerted significantly lower expression in OA synovium of at least three patients following LLLT as compared to controls ($p<0.05$) (Table 3).

Discussion

The clinical efficacy of LLLT in various arthritides including OA has been established. However, little is known about the exact molecular effects of laser irradiation. Some research groups have used gel electrophoresis and MS proteomic techniques to compare the composition of healthy and OA cartilage. Garcia et al. (17) studied the components of OA chondrocytes and extracellular matrix (ECM) by MS method. Several proteins involved in ECM organisation, signal transduction, cell communication, immune responses and metabolism showed differential expression. Kirsch et al. (18) showed the expression of alkaline phosphatase, annexin II and type VI type collagen in the upper zone chondrocytes of human OA cartilage, but none of these in healthy human cartilage.

Here we present only those proteins that exerted significantly increased or decreased expression upon LLLT.

Our results show increased expression of mhsp60 in OA synovial membrane after LLLT compared to untreated controls. This protein belongs to the hsp60 (chaperonin) family (19-21). Tsan et al. (22) consider hsp-s as molecular chaperons recognizing and bounding polypeptid chains, and the partly-folded intermediates of proteins. Hsp-s inhibit aggregation, erroneous folding and directly mediate protein folding as chaperons. Yokota et al. (23) showed that anti-hsp60 antibody levels are higher in autoimmune diseases, such as rheumatoid arthritis, SLE, Sjögren's syndrome and mixed connective tissue disease compared to healthy controls. Sedlackova et al. (24) suggested that the expression profile of hsp60 gene might serve as a new marker in the differential diagnosis between RA and OA. Hsp gene

expression profiles may be very different in inflammatory (RA) and non-inflammatory (OA) joint diseases.

Some other proteins showing diminished synovial expression after LLLT including calpain small subunit 1. This is a calcium-dependent cystein protease formed in the cartilage and synovial tissue. RA and OA chondrocytes actively secrete calpain and calpain levels are increased in both RA and OA synovial fluids compared to normal controls (25, 26). Whiteman et al. (27), as well as Squier et al. (28) described peroxynitrite ($ONOO^-$)-mediated, calcium-dependent mitochondrial dysfunction and apoptosis caused by calpain activation. Szomor et al. (29) found that in murine collagen-induced arthritis (CIA) calpain expression correlated with the severity of inflammation and cartilage destruction. In an early phase of CIA calpain acts as a matrix proteinase and plays a role in enzyme activation and cartilage destruction. Although calpain is regarded as an intraarticular enzyme, recently calpain has also been detected in the site of callus formation after the fracture of growing cartilage (30), as well as in synovial fluids of OA patients (31). Solau-Gervais et al. (32) compared the activities of cystein proteases and dipeptidyl peptidase in synovial tissues of RA, OA and posttraumatic arthritic patients. Calpain activity was higher in RA and OA compared to posttraumatic controls. Struglics et al. (33) suggest that calpain plays a role in the cleavage of human aggrecan. Calpain-generated fragments were detected in both normal and OA cartilage and synovial fluids. Iguchi-Hashimoto et al. (34) described the overexpression of calpastatin, a calpain antagonist in arthritis. Calpastatin supresses IL-6 production, as well as NFκB signalling in RA. As calpain is highly involved in cartilage destruction and its expression decreased by laser irradiation, thus LLLT may diminish calpain-mediated degradation in OA.

Actin and tubulin are cytoskeletal proteins. Tubulin is a member of the family of globular proteins and is a constituent of microtubules. The expression of α-tubulin significantly decreased in all synovial tissue samples following LLLT. Ramos-Ruiz et al. (35) found suppressed tubulin synthesis in the synoviocytes of rats with adjuvant arthritis. The inhibition of tubulin synthesis may be an initial step in the development of adjuvant arthritis.

The expression of cofilin was also reduced by LLLT in two synovial samples in our study. Campbell et al. (36) found that cofilin expression was significantly upregulated in chondrocytes upon mechanical stress. Rollin et al. (37) performed differential proteomic investigation of chondrocytes in OA patients. The expression of cytoskeletal binding proteins including cofilin and annexin 2 significantly increased in OA chondrocytes. Blain et al. (38) described a 3-dimensional network of cytoskeletal tubulin and intermediate filaments. Tubulin

and cofilin are involved in cytoskeleton formation and also in remodelling and mechanical properties of the cartilage.

Vimentin variant 3 expression was significantly reduced after LLLT in the synovial samples of three OA patients. Haudenschild et al. (39) reported that the changes in the organisation of vimentin correlates with the progression of OA, but the results of these changes, especially in chondrocytes are not clear. In their differential proteomic analysis of normal and OA chondrocytes, Lambrecht et al. (40) discovered that the vimentin network is severely impaired in OA. The organization of split vimentin was described in OA chondrocytes. Tilleman et al. (41) studied the cleavage of a citrullinated vimentin isoforms in OA cartilage. Similarly to Haudenschild et al. (39), this group found that the disruption of vimentin network weakened the mechanical integrity of cells. Wang et al. (42) observed that in numerous cell types, the loss of vimentin led to impaired DNA synthesis and cell proliferation. Tilleman et al. (43) concluded: „It could be possible, that a higher expression of vimentin contributes to the invasive character of the synovial pannus like tissue in inflammatory arthritides". Therefore we may suppose, that laser induced decrease of vimentin variant 3 could be antiinflammatory and therefore beneficial in OA.

Ea et al. (44) reviewed the role of annexin A5 in OA. Biphasic calcium phosphate (BCP) and hydroxy-apatite (HA) crystals are associated with severe forms of OA. In severe OA, chondrocyte apoptosis is associated with the overexpression of annexin V and BCP crystal deposition in the matrix. It was demonstrated that the overexpression of annexin V may contribute to hypocellularity of the articular cartilage. The reduction of annexin V expression by LLLT treatment may lead to decreased chondrocyte apoptosis in OA.

We observed a significant decrease in the expression of collagen type VI alpha 2 chain precursor in five OA synovial samples following laser treatment. Hambach et al. (45) demonstrated severe abnormalities in the expression and distribution of pericellular collagen VI in OA. When comparing healthy and OA cartilage, the three chains of collagen VI were abundantly expressed in healthy articular cartilage, while collagen VI expression was significantly reduced in the superficial layer of the cartilage in OA. The authors concluded that the underexpression of collagen VI in the cartilage may result from increased degradation rather than by a decreased synthetic activity. Murray et al. (46) suggested that in OA the disruption of pericellular collagen VI was associated with increased production of IL-1β. Thus, the decrease in collagen VI expression induced by LLLT might be advantageous regarding chondrocyte metabolism.

In conclusion, the significant increase in the expression of mhsp60 after LLLT treatment of OA synovial samples suggests the existence of a reparation process that inhibits protein aggregations and protein misfolding. The decreased expression of calpain small subunit 1, annexin A5, vimentin variant 3 and collagen type VI alpha 2 chain precursor expression after LLLT may result in the suppression of cartilage degradation in OA. Our study provided additional evidence for the molecular effects of LLLT in rheumatic diseases.

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Table 1 Clinical and laboratory parameters of our patients

Patient No.	Age (years)	Disease duration (years)	NSAID treatment
1	76	5	+
2	54	4	+
3	74	5	+
4	73	20	+
5	57	3	-
6	79	5	+

Table 2 Proteins showing significantly altered synovial expression upon laser irradiation

Identification number of spot	Score of protein shots	Mass of protein molecule (kDa)	NCBI ID*	Identified protein
1830	144	28	4502565	calpain small subunit 1
1100	132	61	189502784	mitochondrial heat shock 60kD protein 1 variant 1
1486	94	37	21619816	tubulin alpha-1C
1599	192 150	50 50	5174735 167887751	tubulin beta 2 vimentin variant 3
1803	92	39	4502101	annexin A1
1655	106	36	49168528	annexin A5
1923	92 94	19 23	5031635 48255905	cofilin 1 transgelin
581	94	110	13603394	collagen type VI alpha 2 chain precursor

(* National Center for Biotechnology Information, identification number of the protein at the National Center for Biotechnology (USA))

Table 3 Treated/untreated expression rates of synovial proteins

Identification number of spot	Name of protein	Identification number of patients*	Rate of treated / control	Average of rates
1830	calpain small subunit 1	4 2 1	0.78 0.79 0.67	0.75
1100	mitochondrial heat shock 60kD protein 1 variant 1	2 3 5	6.31 2.00 2.99	3.77
1486	tubulin alpha-1C	6 5 4 3 2 1	0.92 0.87 1.02 0.94 0.84 0.72	0.88
1599	tubulin beta 2 vimentin variant 3	5 3 2	0.58 0.11 0.09	0.26
1803	annexin A1	5 2 6 3 1	0.89 0.90 0.78 0.81 0.87	0.85
1655	annexin A5	2 3 1 4	0.85 0.58 0.75 0.76	0.74
1923	cofilin 1 transgelin	2 3	0.74 0.78	0.76
581	collagen type VI alpha 2 chain precursor	1 5 3 2 6	0.82 0.71 0.77 0.60 0.72	0.72

* Only those patients are indicated in whom there was a significant change ($p < 0.05$) upon laser irradiation .