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Enhanced therapeutic anti-inflammatory effect of betamethasone upon topical administration with low frequency, low intensity (20 kHz, 100 mW/cm²) ultrasound exposure on carrageenan-induced arthritis in mice model

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Abstract

The purpose of this work was to investigate whether low frequency, low intensity (LFLI, 20 kHz, <100 mW/cm², spatial-peak, temporal-peak) ultrasound (US), delivered by a light-weight (<100g), tether-free, fully wearable, battery powered applicator is capable of reducing inflammation in a mouse model of Rheumatoid Arthritis (RA). The therapeutic, acute, anti-inflammatory effect was estimated by the relative swelling induced in mice hind limb paws. In an independent, indirect approach, the inflammation was bio-imaged by measuring glycolytic activity with near infrared labeled 2-deoxy-glucose (2DG). The outcome of the experiments indicated that the combination of US exposure with topical application of 0.1% w/w betamethasone gel, exhibited statistically significant ($p < 0.05$) enhanced anti-inflammatory properties in comparison with the drug or US treatment alone. The present study underscores the potential benefits of LFLI US assisted drug delivery. However, the proof of concept presented indicates the need for additional experiments to systematically evaluate and optimize the potential of, and the conditions for, safe, LFLI ultrasound promoted non-invasive drug delivery.

Keywords

low frequency ultrasound; betamethasone; topical delivery; carrageenan-induced arthritis; anti-inflammatory effect; near infrared imaging

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INTRODUCTION

The purpose of this work was to investigate whether low frequency, low intensity (LFLI is defined here as: 20 kHz, <100 mW/cm², spatial-peak, temporal-peak intensity) ultrasound (US), delivered by a light-weight (<100g), tether-free, fully wearable, battery powered applicator is capable of providing a therapeutic effect in a mouse model of inflammatory arthritis.

The primary motivation for this work was to explore the possibility of syringe-free delivery of topically applied drug using 20 kHz safe (non- thermal and non-cavitation) levels of ultrasound. Such noninvasive delivery is of importance for both societal and economic reasons, because of the adverse reaction or physical inability to administer the drugs orally and reduction in cost of treatment associated with the delivery tools (no syringes needed and no need for controlled disposal of the syringes). Non-invasive delivery is also of interest because oral intake of a variety of drugs might be impeded due to side-effects such as decreased white blood cell counts, increased risk of infections, fatigue, oral ulcers, hair loss, nausea, and diarrhea, and liver toxicity. The pre-clinical evaluation was performed using a mouse model of rheumatoid arthritis (RA). RA was chosen because it is often encountered in children and juveniles. To minimize the side effects pediatric rheumatology treatment usually requires drugs to be injected, which is painful. Also, many children develop such psychological aversion to these injections that they vomit prior to drug administration. Moreover, invasiveness of delivery increases health care costs and reduces patient compliance. In the following, the harmful effects of RA are highlighted.

Rheumatoid arthritis (RA) is a chronic, debilitating inflammatory, musculoskeletal disease that affects 0.5–1.0% of the adult population in Western countries, with total treatment costs approaching \$80 billion annually (Control and Prevention 2006; Lawrence et al. 2008). Its pediatric sibling, Juvenile Idiopathic Arthritis (JIA) affects 0.01–0.4% of children (Manners and Bower 2002). The joint destruction and progressive functional disability associated with uncontrolled inflammation impact ability to work, attend school, overall quality of life, and mortality (Yelin et al. 2007; Cheng et al. 2010).

Systemic Corticosteroids (CS) most often control disease in inflammatory arthritis, RA and JIA. However, if given for a prolonged period, they result in significant morbidities, including glaucoma, cataracts, weight gain, hypertension, diabetes, and bone fragility (Strehl and Buttgereit 2013; Gaujoux-Viala and Gossec 2014). Therefore, alternate systemic agents, are added to spare exposure to steroids. These include: hydroxychloroquine, disease modifying agents (e.g. methotrexate), non-steroidal anti-inflammatory agents, and biologic agents (Grigor et al. 2004; Weinblatt et al. 2010; Nam et al. 2014). These non-steroidal agents have potential side effects including, cytopenias, increased risk of infection, and pain (subcutaneous injections). While biologic agents may be helpful in recalcitrant disease, they typically cost 10 to 100 times more than older therapies, and there has been a global concern regarding their impact on healthcare budgets (Lawrence et al. 2008). These concerns have prompted interest in research and development of novel, economically acceptable, safe delivery methods of existing anti-inflammatory drugs, including CS, to patients with

inflammatory arthritis. The primary goal of these activities is to minimize the side effects of the drugs taken systemically or injected intra-articularly while maximizing their potency in alleviating arthritis.

Intra-articular CS injections (IACI) are frequently used in the management of patients with inflammatory arthritis. They are preferred over systemic agents to treat mono-articular/oligoarticular arthritis (Bigelow et al. 2011). Even in polyarticular disease, the addition of IACI to systemic combination therapy helps achieve “tight control” of RA in a manner that may obviate the need for biologics (Proudman et al. 2000; Grigor et al. 2004; Hetland et al. 2006)). While studies have demonstrated, at least in children, that triamcinolone hexacetonide (with the lowest solubility) is the most effective (Zulian et al. 2004), clinical effect has been demonstrated with methylprednisolone, betamethasone, triamcinolone acetonide, and triamcinolone hexacetonide IACI (Sherry et al. 1999). IACI have rapid onset, often within the week. Following IACI, arthritis is controlled in 63–82% of children at 6 months, 45%–50% at 12 months in JIA (Kovar et al. 2009; Ziskin 2010). However, IACI are acutely painful; while not in adults, in children they often result in the need for sedation. Moreover, they can result in osteoporosis (Laan et al. 1999), skin hypopigmentation, subcutaneous atrophy, or rarely introduce infection into the joint (Gilsanz and Bernstein 1984; Charalambous et al. 2003). Although intra-articular administration of CS derivatives can result in rapid onset of a durable anti-inflammatory response that may limit or obviate exposure to systemic medications (Proudman et al. 2000; Conaghan et al. 2003; Grigor et al. 2004; Hetland et al. 2006), alternative effective but non-invasive ways to deliver local CS are needed. The fully wearable, battery operated ultrasound applicator described here alleviates these issues. In particular, as already noted, it might provide a painless route of treatment, while minimizing side effects. It also offers a potential for home treatment without the need to visit the physician’s office. This, in turn, will potentially enhance the patient’s quality of life and by eliminating the need for transportation to the hospital, further lessen the cost of treatment.

The research in ultrasound assisted drug delivery has gained increasing attention over the past two decades. While there is bevy of publications on the topic, here, for brevity and convenience, only one extensive and systematic review describing the current knowledge and state-of-the-art of US assisted drug delivery is highlighted. This recent, well-documented (with 181 references) survey was published by the team of researchers from Ben Gurion University, Beersheba, Israel (Azagury et al. 2014). The information provided therein (Azagury et al. 2014) should be helpful in facilitating an insight into the encouraging outcome of the *in vivo* animal study described in the following.

In the Materials and methods section, the ultrasound applicator used in this study is presented along with all relevant exposure parameters. In addition, the conditions of the *in vivo* mice experiments are described. Next, the results indicating the potential of the LFLI ultrasound assisted transdermal drug delivery are outlined and followed by the discussion and conclusions.

MATERIALS AND METHODS

Prior to description of the applicator used in the experiments described in the following, it might be useful to elucidate the choice of the US field parameters employed in this study.

The initial literature review suggested that US at frequencies in the tens of kHz range might be more effective for noninvasive, transdermal drug delivery in comparison to the therapeutic 1–3 MHz interval. This notion was also supported by the outcome of our independent study (Pong et al. 2006) in which 20 kHz US caused more liposome membrane disruption than 1 MHz and 1.6 MHz ultrasound. These findings were further supported by Sundaram, Mellein and Mitragotri (Sundaram et al. 2003); they reported that exposure to 20–100 kHz range US caused increased fibroblast membrane permeability providing a defined biological effect which may be relevant to the therapeutic effects of US.

The design of the ultrasound applicator (Sunny et al. 2012) used in the current experiments is described below.

Ultrasound applicator

The applicator (Sunny et al. 2012; Samuels et al. 2013) operated at 20 kHz and delivered 50% duty cycle, 55 kPa peak-to-peak pressure amplitude corresponding to 100 mW/cm² spatial-peak, temporal-peak, intensity (I_{SPTP}). To optimize battery lifetime (between re-charging) pulse repetition frequency was chosen to be 25 Hz (Samuels et al. 2013). An early prototype of an ultrasound applicator used in the experiments described in the next section is shown in Fig. 1, along with the compact box containing electronic circuitry including custom-built tone burst generator powered by rechargeable lithium-ion batteries. A detailed description of construction, principle of operation and characteristics of the device was given in (Sunny et al. 2012). The lightweight (< 25 g), small foot print (approximately 20 mm × 20 mm × 8 mm) applicator was designed to be used as a tether-free, fully portable tool. As noted earlier, the applicator was driven by a 20 kHz tone-burst sinusoidal excitation waveform with a 20 ms pulse duration delivered at a PRF (pulse repetition frequency) of 25 Hz. The applicator was activated using a custom designed battery-operated driving unit. The heart of the applicator is a piezoelectric ceramic disk (PZT 4), sandwiched between the metal caps, and the whole flexural element is 2–3 mm in height. Typically, the thickness of the capacitive piezoelectric element is inversely proportional to the frequency, and where a 1 MHz (typical therapeutic frequency range) element is about 2 mm in thickness, a 20 kHz element would need to be 10 cm thick. Fundamental thickness operation of such an element would require excitation on the order of 100's of volts, adding bulk and removing any chance of being a portable design. The design was implemented as a mechanical displacement amplifier, which, much like a gear box can scale movement up or down, translates 2 MHz US waves into 20 kHz output at the desired pressure amplitude of 55 kPa (this amplitude corresponds to spatial-peak, temporal-peak intensity level of 100 mW/cm², I_{SPTP}) with only 12–15 volts excitation. Without this technique, a large (and heavy, typically well above 10 kg) power amplifier would be needed to drive the highly capacitive piezoelectric ceramic load.

The acoustic output of the applicator was measured in 22 °C de-ionized water, using a calibrated hollow cylinder hydrophone, with a diameter of 2 mm (Lewin and Chivers 1981a). The two-dimensional (2D) pressure amplitude map of the applicator's acoustic output distribution is shown in Fig. 2. The pressure measurements were performed at a distance of 2.5 mm away from the transmitting surface of the transducer (to mimic a clinical application). For the sake of the discussion it is appropriate to underscore that the applicator's pressure amplitude was set to 55 kPa (corresponding to 100 mW/cm² I_{SPTP}) to – intentionally – rule out inertial cavitation, a phenomenon where gas bubbles violently implode and may cause tissue damage (Nyborg 2001). This pressure amplitude is also considered safe even at prolonged (>250 minutes) exposure time, according to the 2008 Statement on Mammalian In-vivo Ultrasonic Biological Effects from the American Institute of Ultrasound in Medicine (AIUM 2008).

Two independent tests confirmed that the applicator does not produce inertial cavitation. The first test used aluminum foil to show pitting if inertial cavitation was present. The second used a hollow cylinder hydrophone (Lewin and Chivers), coupled to a HP 8535A Spectrum Analyzer to display the cavitation spectrum. This technique allowed both stable and inertial cavitation events to be determined. Stable cavitation manifests itself by generation of subharmonics and its odd multiples (Eller and Flynn 1968), whereas inertial cavitation is characterized by white noise produced by the imploding of different diameter microbubbles (Datta et al. 2006). In both experiments, the applicator showed no signs of inertial cavitation. These outcomes are further corroborated by the literature data, where the inertial cavitation pressure amplitude threshold in water at 20 kHz was reported to be on the order of 100 kPa (Apfel 1981), and was dependent on the medium properties including cavitation nuclei sizes and bubbles' concentration. Clearly, this threshold is higher than the 55 kPa peak-to-peak pressure amplitude used here in the exposure studies described in the next section.

Independently, using thermocouple measurements it was determined that at the 100 mW/cm² SPTP intensity level used the applicator did not experience any self-heating and the temperature elevation in the immediate vicinity of the skin was below 0.5 °C even at prolonged - up to 45 minutes - exposure. The paw temperature was recorded with a Testo 735 m (Testo AG, Germany) using a probe TC type K. The probe was placed in contact with the paw of the mouse and the temperature recorded.

RA carrageenan mice model and ultrasound exposure

All experiments described below were performed at 20 kHz with the applicator operating at pressure amplitude of 55 kPa (peak-to-peak), with duty cycle of 50%, pulse repetition frequency of 25 Hz and total exposure time of 20 minutes. The topical application of the drug contains 0.1% w/w betamethasone (Sigma-Aldrich, St Louis, MO, USA) in a water soluble polyethylene gel. In controls the same gel without the drug was used.

The anti-inflammatory drug betamethasone was chosen for this feasibility study because it represents the long acting corticosteroids with half-life of 36–54 hr (Swartz and Dluhy 1978); hence a single dose used was adequate for the duration of the experiments described. Also, this compound is very potent and allows lower dose to be employed. For example 0.7

mg betamethasone is equivalent to 29 mg of the short acting cortisol. Finally, a lower volume (dose) of betamethasone was much easier to dissolve in the aqueous gel used as a coupling medium between the ultrasound applicator (Fig. 1) and the animal's (mouse) limb.

Male ICR (CD-1®) (a Swiss mouse species) mice received topical administration of betamethasone combined with 20 min exposure to ultrasound (n=11), 1 hour prior to carrageenan injection (Figure 3). The betamethasone/ultrasound treatment was performed before the carrageenan induction due to the fact that the animal model was used - in this model to generate anti-inflammatory effect the compound/treatment has to be delivered prior to carrageenan injection. This is in contrast to the human patient's situation: in a true diseased patient, the arthritis would already be present before treatment was administered. Overall, designing a protocol for therapy of inflamed joint, especially using animal models is not straightforward and deserves another dedicated study. Control mice received topical administration of betamethasone (n=11), same 20 min exposure of US (n=11) or were left untreated (n=16). Three (3) mg/kg carrageenan (Sigma-Aldrich, St Louis, MO, USA) was injected into the sub plantar area of the mice left hind paws. Hind paw thickness was measured by JOCAL (Mitutoyo, Japan) calipers calibrated in 0.01 mm gradations before carrageenan injection and afterward at intervals of 1 h between each measurement. Swelling was calculated as the difference between the thickness of the hind paw measured at each time interval and its size at zero time (before carrageenan injection). The data were presented as the difference between mean thickness of the hind paw measured (by micrometer) at each time interval to its mean size at time zero (before carrageenan injection). The caliper method to assess the degree of swelling was reported previously (Lodzki et al. 2003; Hussein et al. 2012). Although there are alternative quantitative methods capable of determining the swelling volume, in general they are much more complex: one would require using 3 Tesla MRI (Borrero et al. 2011); another the use of specialized measurement tool such as a plethysmograph (Govindarajan et al. 2007). The caliper method was chosen here as it provided adequate information about relative swelling induced by the carrageenan and was considered sufficient for this feasibility study.

***In vivo* NIR imaging of paw inflammation in carrageenan-induced arthritis mice model**

To obtain an independent verification of the therapeutic effect of betamethasone formulation exposed to ultrasound, near infrared imaging scans were performed. To this end, one hour after carrageenan injection, 10 nM of IREDye 800CW 2DG (LI-COR Biosciences, Lincoln, NE, USA) was injected intravenously into the tail of all groups of mice to measure glycolytic activity. *In vivo* imaging of IREDye 800CW 2DG fluorescence in mice was performed with a LI-COR Biosciences small-animal imager Odyssey MousePOD® (LI-COR Biosciences, Lincoln, NE, USA) with the following conditions: resolution: 170–340 microns; pixel area: 0.03 mm² (approximately 15–20 cells); quality: medium-low; focus offset: 1–3; channels: 800 nm; intensity: 1–3. Mice received topical administration of betamethasone combined with 20 min ultrasound exposure (n=4), 1 hours prior to carrageenan injection. Again, control mice received topical administration of betamethasone (n=3), 20 min ultrasound exposure (n=4) or were left untreated (n=4). 3 mg/kg carrageenan was injected into the sub-plantar area of the mice left hind paws. One hour after the carrageenan injection, mice were injected with 10 nmol of IREDye 800CW 2DG via the tail

vein. Images were taken 2 hours after the optical imaging probe, IRDye 800CW 2DG, injection. The NIR images were processed using applied spectral imaging software, Spectral View™ (ASI, Migdal Ha'Emek, Israel) (Cohen et al. 2012). Intensity scale: red, green and blue denotes high, intermediate and low signal intensity respectively. As already pointed out, the ultrasound exposure did not generate any thermal effect, hence it has not influenced the NIR imaging results. However, it might be instructional to explain the informed choice of the above mentioned dye (IRDye 800CW 2-DG) as the fluorescent agent.

Briefly, the use of noninvasive NIR tool for visualization of the experimental data presented in Fig. 4 was implemented by recognizing the fact that inflammation, similarly to the malignant tumors or cancers manifests itself as high metabolic activity and leads to elevated rate of glycolysis, even under aerobic conditions. Thus, it is well suited for diagnosis purposes because the increased demand for glucose to fuel energy production in inflammation or in tumors translates to elevated expression and activity of cell surface glucose transport proteins, which can then be exploited for noninvasive imaging using glucose analogs. These findings have been applied extensively in diagnostic clinical imaging employing positron emission tomography (PET), in which ¹⁸F-DG (2-fluoro-2-deoxyglucose) uptake is used to visualize tumor activity and location. Whereas the method is sensitive and quantitative and has been used in many pre-clinical studies, the use of ¹⁸F-DG is impractical due to the specialized detection instrumentation required and relatively short (110 minute) half-life of the isotope. Therefore, in this study the ¹⁸F-DG radionuclide was replaced by near infrared fluorescent glucose analogue, IRDye 800CW 2-DG.

Statistical analysis

The data is presented as mean $\pm$ SD (Standard Deviation) of large series of independent experiments using different batches of 12–17 of carrageenan-induced arthritis mice in each experiment performed during a period of two years. The experiments were repeated 4 to 6 times. All data were evaluated using the InStat statistics program (GraphPad, La Jolla, CA, USA). Statistically significant differences between experimental groups were determined by analysis of variance (ANOVA) with Bonferroni post-hoc test and considered significant when $p < 0.05$.

RESULTS

Anti-inflammatory effect of low frequency ultrasound combined with betamethasone

Figure 3 presents the typical outcome of the experiment, summarizing the comparison between groups of 10–11 mice with carrageenan-induced arthritis, which received topical administration of betamethasone with and without ultrasound and respective controls. The results indicate that the administration of US with 25 mg betamethasone per kg mice, has a significant ($p < 0.05$) therapeutic effect in comparison with the untreated group or either US alone or drug alone treated groups.

Bio-imaging of the anti-inflammatory effect of low frequency ultrasound combined with betamethasone

The outcome of the quantitative and qualitative analysis of paw glycolic activity in carrageenan-induced arthritis in mice model is shown in Fig. 4. The infrared imager images were processed using Applied Spectral Imaging Software, Spectral View. High intensity signal is shown in red; intermediate one in green and low is shown in blue. The NIR intensity was estimated at identical conditions for all mice. The statistically significant differences ($p < 0.05$) were found between combined ultrasound and betamethasone treated and untreated mice. Interestingly, ultrasound treatment alone also provided a statistically significant ($p < 0.05$) beneficial therapeutic effect when compared with untreated mice.

DISCUSSION

The present study indicates that low frequency, low intensity ultrasound can assist in topical delivery of anti-inflammatory RA drugs such as betamethasone. In this study, an ultrasound aided enhancement of betamethasone delivery was observed. However, the proof of concept presented in this pilot study indicates the need for additional experiments systematically evaluating and optimizing the potential and efficacy of delivery.

Optical fluorescence imaging is increasingly being used to monitor tissue inflammation. Tissues under physiological conditions are labeled by 2DG dye at background levels. By contrast, the elevated rates of glycolysis and glucose transport in the cells undergoing an inflammatory process provide a clearly visible fluorescent signal by comparison to non-inflamed tissue. This approach was employed here to visualize the glycolytic activity on the paws of the carrageenan-induced arthritis model using 2-DG labeled with a near infrared probe (Kovar et al. 2009; Cha et al. 2012). The outcome of these experiments further support the significant therapeutic effects of combined ultrasound exposure and betamethasone topical application by comparison to topical drug treatment administration alone, as measured by glycolytic activity.

The common clinical rationale for treating arthritic joints with drugs by intra-articular injection is that therapeutic concentrations of drug that can be attained in the joint compartment using a minimal total dose of betamethasone per joint thus resulting in minimal systemic exposure and toxicity. For example, in a rat model of collagen induced arthritis (Arsenault et al. 2000) rats were administered intravenously a total dose of 52 mg/kg paclitaxel in six divided doses over ten days. In another study (Liggins et al. 2004) using the same model in rabbits, but using a slow release microsphere formulation, the dosage was 1.4 mg/kg. Thus, the dose given to rabbits was approximately forty-fold lower than the systemic dose given to rats in the collagen induced arthritis model (Liggins et al. 2004). Furthermore, it has also been shown that in healthy rabbits injected intra-articularly with either free methotrexate or controlled release methotrexate loaded microspheres, the plasma levels of methotrexate were 10-fold lower in rabbits administered methotrexate microspheres compared to free methotrexate (Liang et al. 2004). Wigginton et al. (Wigginton et al. 1980) showed that following a 5 mg intra-articular dose of methotrexate solution in rheumatoid arthritis patients, synovial fluid levels were between 10 to 250-fold higher than serum levels over the first 24 h. Similarly, in the present study the combination

of ultrasound exposure with 25 mg betamethasone per kg mice are anti-inflammatory, at least in the short term, in comparison to drug treatment alone, which was not effective at the same dosage. It is anticipated that a further analysis of the betamethasone release will be needed specifically in terms of pharmacokinetic evaluations of bethamethasone maintenance in the rat intra-articular synovial fluid.

Despite the limitations (side-effects) of systemic administration of anti-rheumatic drugs, intra-articular delivery of disease modifying anti-rheumatic drugs has not attracted much interest so far because these drugs were not expected to remain in the joints long enough to be locally effective (Bragdon et al. 2001). Delivery of drugs in joints using LFLI ultrasound promoted non-invasive drug delivery offers an exciting alternative for a novel intra-articular drug delivery method. Although it is expected that by this approach less drug will be delivered, frequent administrations, which are less painful than injections, may provide the cumulative therapeutic dose. The *in vivo* results presented here obtained in antigen-induced acute arthritis are sufficiently encouraging to warrant further investigation in chronic models. It is also conceivable that the protocol or paradigm rate of treatment is of importance, and a rate different than the one used here would potentially further enhance the efficacy of the treatment.

The following open questions remain ensuing the present pilot study: i. Is the anti-inflammatory effect seen here due to drug or the ultrasound (US)? ii. Can US get drug through the much thicker synovium of chronically, rather than acutely, inflamed joints; iii. Recognizing that betamethasone might not be the optimal drug, could other Cortico-Steroids (CS) be delivered by the same protocol? iv. As US delivery of CS can be administered more frequently than the intra-articular CS injections, might betamethasone then be preferable despite its shorter half-life? v. Might ultrasound treatment result in unanticipated side effects? With respect to that, our own analysis mentioned in the previous sections indicated that this is very unlikely at the exposure levels used in this work. In addition, the review presented in (Azagury et al. 2014) appears to suggest that the safety of the US assisted drug delivery is commonly accepted. On the other hand, whereas the information provided in (Azagury et al. 2014) indicates that the US assisted drug delivery is achievable, the biological end-points observed are not straight forwardly linked to all relevant ultrasound field parameters, such as its pressure amplitude or intensity, rate of delivery, frequency and total exposure time.

Of particular interest is the mechanism(s) of action, as its knowledge would permit personalized treatment and healthcare cost reduction. Ultrasonically assisted drug delivery mechanisms proposed in the scientific literature (Azagury et al. 2014) include mechanical and thermal effects, heat generated as a result of the ultrasound energy propagating in lossy medium, such as skin tissue. As confirmed by the thermocouple measurements described above, no temperature elevation was observed. However, to facilitate comparison with diagnostic imaging conditions, the Thermal Index (TI - soft tissue case) was calculated using the guidelines of (Bigelow et al. 2011) indicating the TI value equal to 0.01. The corresponding thermal dose index was calculated to be 0.237 (Ziskin 2010). With the mechanical effects associated with inertial cavitation (sonophoresis) ruled out (see Methods section), it is appropriate to reiterate that in the study described here the transdermal drug

delivery was most likely due to nonthermal, non-inertial cavitation effects such as acoustic radiation forces and stable cavitation.

Acoustic radiation forces are mechanical stresses caused by acoustic energy interacting directly with cells (Nyborg 2001), whereas stable cavitation indirectly induces mechanical stress by setting up acoustic streaming, which is a steady state fluid flow induced by time variable ultrasound energy (Rooney 1972; Lewin and Bjørnø 1982). Additional mechanisms such as change in tissue architecture and change to cell membrane permeability might be considered. However, it is also plausible that the changes in permeability are not necessarily due to the direct effect of a mechanical (ultrasound induced force) enforcement but encompass more intricate, multi-step mechanism involving mechanoreceptors and chain-reaction on the cellular level. It is further conceivable that the ultrasound mechanical stimulation activates cellular components such as macrophages, fibroblasts or keratinocytes and it is their “cross-talk” that results in changes in cell membrane permeability. Finally, the molecular weight of the drugs will need to be scrutinized and their chemical properties defined in order to qualify them as suitable candidates for transdermal delivery using the ultrasound exposure conditions considered in this work. Whatever the mechanism responsible, this study underscores the potential benefits of low frequency, low intensity, ultrasound assisted corticosteroid delivery in an acute model of RA in mice.

Again, it may be useful to underscore that the pressure amplitude level of 55 kPa (corresponding to spatial-peak, temporal-peak intensity of 100 mW/cm²) and used in this study, rules out inertial cavitation and thermal effects as possible mechanisms of action, leaving radiation force and stable cavitation with associated second order acoustic phenomenon, such as acoustic micro-streaming (Nyborg 2001). In this context it is also conceivable that the model (which does not require pre-existence of tiny air voids) proposed recently by (Krasovitski et al. 2011) might be helpful to explain the results described. Krasovitski et al. (Krasovitski et al. 2011) proposed cellular structure referred to as bilayer sonophore, which represented dynamic behavior of the two lipid bilayer membranes that was separated by hydrophobic volume. Exposed to ultrasound field this volume changes together with the compressional and rarefactional phases of the field. Negative pressure amplitude allows the distance between the layers to increase whereas the positive one brings them closer to each other. The modeling shows that the sonophore alone (without the need for existence of tiny air voids) is capable of transforming acoustic field into mechanical forces acting at the (sub) cellular levels.

In conclusion, the findings of this study indicate that the combination of ultrasound exposure delivered with a special applicator together with betamethasone topical application are anti-inflammatory under acute conditions of therapy in contrast to drug or ultrasound treatments alone. Quantitative bio-imaging of anti-inflammatory effect in the left paw of the carrageenan injected mice clearly confirmed the therapeutic effect of US-betamethasone formulation.

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Abbreviations

2DG	2-deoxy-glucose
CS	corticosteroids
IACI	intra-articular corticosteroid injection
JIA	Juvenile idiopathic arthritis
LFLI	low frequency low intensity
NIR	near infrared
PRF	pulse repetition frequency
PZT	piezoelectric ceramic disk
RA	rheumatoid arthritis
SPTP	spatial-peak, temporal-peak
US	ultrasound

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Figure 1.
Single element 20 kHz ultrasound applicator used in this study along with the compact box containing electronic circuitry for exposure control and application on mice limbs.

Figure 2. Two-dimensional representation of the pressure field distribution on the paw, measured at an axial distance of 2.5 mm from a single element applicator

Two-dimensional beam plot showing uniform ultrasound field distribution of the flexural ultrasound applicator at the axial distance of 2.5 mm. Normalized two-dimensional surface pressure amplitude distribution measured at the axial distance of $z = 2.5$ mm of the applicator having characteristics shown in Fig. 1a. Y axis: left to right direction, X axis: front to back direction, intensity: violet (higher pressure amplitude, higher voltage) to red (lower pressure amplitude, lower voltage). Maximum pressure amplitude measured at the axial distance of 2.5 mm was 55 kPa (100 mW/cm^2). The pressure amplitude decreases by a factor of 3 dB at the axial distance of 8 mm.

Figure 3. Effect of low frequency US combined with betamethasone topical administration on carrageenan-induced arthritis in a mice model

Male CD1 mice received topical administration of 25 mg/kg mice betamethasone combined with 20 min exposure of low frequency US (n=11), 1 hour prior to carrageenan injection. Control mice received topical administration of 25 mg/kg mice of betamethasone (n=10), 20 min exposure of low frequency US (n=10) or left untreated (n=11). Three (3) mg/kg carrageenan was injected into the sub-plantar area of the mice left hind paws. * p<0.05 compared no treatment group; ** p<0.05 compared to betamethasone treated group; *** p<0.05 compared to US treated group.

Figure 4. Near infrared fluorescent imaging of paw glycolic activity in carrageenan-induced arthritis in mice model

One hour after the carrageenan treatments previously described, mice were injected with 10 nmol of IRDye 800CW-2 DG via the tail vein. Images were taken 2 hours after the optical imaging probe, IRDye 800CW-2 DG, injection via the tail vein. The infrared imager images were processed using Applied Spectral Imaging Software, Spectral View. Intensity scale: high intensity signal is shown in red; intermediate intensity signal, green; low intensity signal blue. The NIR intensity was estimated at identical conditions for all mice. * $p < 0.05$ compared no treatment group; ** $p < 0.05$ compared to betamethasone treated group; *** $p < 0.05$ compared to US treated group.