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Nonthermal Effects of Therapeutic Ultrasound: The Frequency Resonance Hypothesis

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Objective: To present the frequency resonance hypothesis, a possible mechanical mechanism by which treatment with nonthermal levels of ultrasound stimulates therapeutic effects. The review encompasses a 4-decade history but focuses on recent reports describing the effects of nonthermal therapeutic levels of ultrasound at the cellular and molecular levels.

Data Sources: A search of MEDLINE from 1965 through 2000 using the terms *ultrasound* and *therapeutic ultrasound.*

Data Synthesis: The literature provides a number of examples in which exposure of cells to therapeutic ultrasound under nonthermal conditions modified cellular functions. Nonthermal levels of ultrasound are reported to modulate membrane properties, alter cellular proliferation, and produce increases in proteins associated with inflammation and injury repair. Combined, these data suggest that nonthermal effects of therapeutic ultrasound can modify the inflammatory response.

Conclusions: The concept of the absorption of ultrasonic energy by enzymatic proteins leading to changes in the enzymes activity is not novel. However, recent reports demonstrating that ultrasound affects enzyme activity and possibly gene regulation provide sufficient data to present a probable molecular mechanism of ultrasound's nonthermal therapeutic action. The frequency resonance hypothesis describes 2 possible biological mechanisms that may alter protein function as a result of the absorption of ultrasonic energy. First, absorption of mechanical energy by a protein may produce a transient conformational shift (modifying the 3-dimensional structure) and alter the protein's functional activity. Second, the resonance or shearing properties of the wave (or both) may dissociate a multimolecular complex, thereby disrupting the complex's function. This review focuses on recent studies that have reported cellular and molecular effects of therapeutic ultrasound and presents a mechanical mechanism that may lead to a better understanding of how the nonthermal effects of ultrasound may be therapeutic. Moreover, a better understanding of ultrasound's mechanical mechanism could lead to a better understanding of how and when ultrasound should be employed as a therapeutic modality.

Key Words: immunology, injury, signal transduction, molecular mechanism, wound healing, cytokines

Ultrasound has become a common therapy for a number of clinical conditions: sprained ligaments, inflamed tendons and tendon sheaths, lacerations and other soft tissue damage, scar tissue sensitivity and tension, varicose ulcers, amputations, neuromata, strained and torn muscles, inflamed and damaged joint capsules, fasciitis, and delayed-onset muscle soreness.[1,2] Recent uses include the accelerated healing of fractures,[3–5] muscle injury,[6] and thrombolysis.[7–16]

Over the past several years, research investigating the cellular and molecular effects of nonthermal levels of ultrasound has accumulated. While clinicians state that ultrasound is used to accomplish heating within deep tissue, there is a common, whispered belief that heating alone cannot account for the clinical effects, especially when ultrasound is delivered at nonthermal settings. My purpose is to review the past 4 decades of ultrasound research and to propose a molecular mechanism whereby the mechanical properties of ultrasound interact with the molecular and multimolecular complexes within the cell. The frequency resonance hypothesis incorporates past research demonstrating ultrasound's mechanical properties (absorption, cavitation, acoustical streaming) with current knowledge within the field of cellular and molecular biology, specifically the activation of proteins and signal-transduction pathways that may result in modifications to cellular function.

Thermal Effects of Ultrasound

Ultrasound is capable of producing thermal therapeutic effects.[2] In 1987, Dyson[1] suggested that the tissue must reach a temperature of 40°C to 45°C for at least 5 minutes to be therapeutic in nature. Experiments performed with nonperfused tissue demonstrated that ultrasound could increase the tissue temperature at a rate of 0.86°C/min (1 W/cm^2, 1 MHz).[17] However, the results of these experiments were difficult to interpret because they were performed in nonperfused tissue. In living tissue, as the temperature increases, the normal blood flow to the area dissipates the heat. More recent, direct in vivo measurement of tissue temperature during ultrasound treatment has resolved the question of tissue heating.[18–21] Draper et al,[18,19] Ashton et al,[20] and Chan et al[21] inserted thermistors to various depths (5 cm or less) and measured the increase in muscle temperature during a 10-minute treatment with either 1-MHz or 3-MHz ultrasound. The data show that treatment

with 1-MHz or 3-MHz ultrasound resulted in a time- and dose-dependent increase in tissue temperature.[18–21] The 3-MHz frequency increased tissue temperature at a faster rate than the 1-MHz frequency.[19] More recently, Ashton et al[20] and Chan et al[21] employed similar techniques to study increases in temperature in the patellar tendon and the effects of coupling media on increases in tissue temperature. While a number of questions remain unanswered with respect to the thermal effects of ultrasound, the purpose of my review is to focus on the nonthermal effects of ultrasound. I will not include the various therapeutic applications of ultrasound that have recently been reviewed elsewhere.[22]

Nonthermal Effects of Ultrasound

A number of experimental designs appear to have successfully isolated the nonthermal from the thermal effects of ultrasound within cellular systems.[1,2,23–25] In vivo, a portion of the energy from the ultrasound wave is absorbed into the tissue structure and converted into heat energy.[2,24] The amount of heating is determined by the frequency and intensity of the ultrasound (dosage) and the type of tissue that is exposed to acoustic energy. A 1982 report demonstrated a direct relationship between the absorption of ultrasound and amount of protein.[26] More simply, as the concentration of protein increased, the absorption of ultrasound increased. In normal tissue, the absorption of ultrasound energy varies depending on the amount of protein in the tissue.[26] In 1980, Love and Kremkau[23] demonstrated that by eliminating extracellular tissue structures (collagen, fibrin, elastin, etc) and placing only the cells in tissue culture media maintained at 37°C, they could treat cells at therapeutic levels without significant increases in temperature (less than 0.5°C over a 10-minute exposure). Our data confirm that cell cultures treated with either 1-MHz or 3-MHz ultrasound at intensities of 0.5 W/cm^2 sustained less than 0.5°C increases over a 10-minute exposure (unpublished observation, 1998). At first it may seem that these data are contradictory to those of Draper et al,[18,19] Ashton et al,[20] and Chan et al[21]; however, the 2 experimental protocols were significantly different. The in vivo measurements performed by Draper et al,[18,19] Ashton et al,[20] and Chan et al[21] recorded actual increases within intact muscle and tendon. The tissue culture protocol eliminates the extracellular structural proteins (collagen, fibrin, elastin, etc)[23,26] that are responsible for most of the increase in temperature observed within the intact tissues.[18–21] Moreover, the tissue culture protocol makes it possible to ''eliminate'' the thermal effects of ultrasound and to study the mechanical effects of ultrasound in an attempt to identify a mechanical mechanism of action.

While exposure of single cells to ultrasound does not increase the overall temperature of the experimental system,[23] it is difficult to determine whether larger temperature increases occurred at the cell surface or within the microenvironments of the cell. Theoretically, larger increases in temperature could occur within microenvironments of the cell as a result of cavitation.[24] However, direct measurements of these types of microenvironmental changes in temperature are currently not possible.

Therapeutic ultrasound produces a combination of nonthermal effects (acoustic streaming and cavitation) that are difficult to isolate. Acoustic streaming is defined as the physical forces of the sound waves that provide a driving force capable of displacing ions and small molecules.[24] At the cellular level, organelles and molecules of different molecular weight exist. While many of these structures are stationary, many are free floating and may be driven to move around more stationary structures. This mechanical pressure applied by the wave produces unidirectional movement of fluid along and around cell membranes.[25]

Cavitation is defined as the physical forces of the sound waves on microenvironmental gases within fluid. As the sound waves propagate through the medium, the characteristic compression and rarefaction causes microscopic gas bubbles in the tissue fluid to contract and expand. It is generally thought that the rapid changes in pressure (caused by the leading and lagging edges of the sound wave), both in and around the cell, may cause damage to the cell. Substantial injury to the cell can occur when microscopic gas bubbles expand and then collapse rapidly, causing a ''microexplosion.'' Although true microexplosions, referred to as unstable cavitation, are not thought to commonly occur at therapeutic levels of ultrasound, pulsation of gas bubbles may disrupt cellular activity, altering the function of the cell.[27]

Early studies investigating the gross effects of acoustic streaming and cavitation on cells showed growth retardation of cells in vitro,[28–31] increases in protein synthesis,[32,33] and membrane alterations.[34,35] Combined, these results may suggest that ultrasound first ''injures'' the cell, resulting in growth retardation, and then initiates a cellular recovery response characterized by an increase in protein production. These findings encompass both continuous and pulsed ultrasound at therapeutic levels ranging from 0.1 to 1.7 W/cm^2.[28–35]

Attraction of Immune Cells to the Injured Area

The natural course of tissue injury can be categorized into 4 distinct phases: acute inflammation, clearance of tissue debris, cellular proliferation, and tissue remodeling.[1,36]

With the early arrival of immune cells to the injured tissue, the immune system can be destructive in nature. When soft tissue is injured, platelets and mast cells are activated and release chemokines, attracting polymorphonuclear cells and blood monocytes (macrophages). Once activated, macrophages produce a unique set of proteins that aid in the destruction of damaged tissue and attract additional lymphocytes to the area. A concerted recruitment of lymphocytes is accomplished by the production of chemokines and the activation of adhesion molecules on the surface of the local capillaries. Adhesion molecules can be viewed as docking proteins that grab circulating lymphocytes and aid in their migration to the injured tissue.[37,38] Reports[39–42] suggest that the nonthermal effects of ultrasound aid the immune response by inducing vasodilation of arterioles and activation of adhesion molecules. Both vasodilation and the activation of adhesion molecules are regulated by signal-transduction pathways,[37–42] suggesting that the ultrasound treatment modified cellular activity by modulating one or more signal-transduction pathways. In general, signal-transduction pathways are composed of a series of enzymatic proteins that are turned on and off by the addition and deletion of phosphate molecules. Phosphate modifications to a molecule lead to distinct changes in conformation (3-dimensional configuration) and regulate the enzymatic activity of protein. A simple analogy for changes in 3-dimensional shape altering function is a pocketknife. When the knife is open, the blade is functionally available and can cut; however, when the knife is closed, the blade is functionally not available. Similarly,

Cellular and Molecular Effects of Nonthermal Ultrasound on Wound Healing

Increase in Protein or Cellular Function	Producing Cell Type	Effector Function
Interleukin-1β[45]	Osteoblasts, monocytes	General inflammatory mediator
Interleukin-2[48]	T cells	T-cell growth
Interleukin-8[45,50]	Osteoblasts	Endothelial cell migration and proliferation
Vascular endothelial growth factor[45,50]	Osteoblasts, monocytes	Endothelial cell migration and proliferation
Basic fibroblast growth factor[45]	Osteoblasts	Endothelial cell migration and proliferation
Fibroblast growth factor[50,53]	Monocytes	Fibroblast growth
Collagen[5,45]	Osteoblasts, fibroblasts	Wound healing
Chloramphenicol acetyl transferase[56]	HeLa, NIH/3T3, C1271	Gene expression of liposomal transfection
Increased proliferation[45]	Fibroblasts	Enhanced wound healing
Increased proliferation[45]	Osteoblasts	Enhanced wound healing
Lymphocyte adhesion[41]	Endothelial cells	Enhanced lymphocyte trafficking
Vasodilation[39,40,42]	Capillary, endothelium	Enhanced blood flow

proteins have an ''active site'' that can be either available or not available, depending on the 3-dimensional shape of the protein.

Inflammatory Response, Injury Repair, and Therapeutic Ultrasound

Later in the inflammatory process, immune cells alter their course of action, aiding in the clearance of tissue debris and stimulating tissue remodeling. This pivotal action is directed by cytokines. For example, the arrival of T cells in an injured area may enhance the immunologic response by releasing T-cell growth factors (interleukin [IL]-2 and IL-4) and immunoregulatory cytokines (IL-10 and interferon-γ).[43] At a certain point in the immune intervention, anti-inflammatory cytokines (largely transforming growth factor-β) are either produced or activated. These anti-inflammatory cytokines down-regulate T-cells and redirect the cellular activities toward proliferation of fibroblasts, collagen production, and remodeling of the damaged tissue.[36,44]

A number of reports have demonstrated that ultrasound affects cells that are centrally involved in the immune response. Specifically, ultrasound has been shown to modulate vasoconstriction; lymphocyte adhesion properties of endothelium, mast cell degranulation, phagocytosis by macrophage, production of growth factors by macrophages; calcium fluxes in fibroblasts; angiogenesis; proliferation of T-cells, osteoblasts, fibroblasts, and a number of proteins associated with inflammation and repair (IL-1, IL-2, IL-6, IL-8, interferon-γ, fibroblast growth factor-b, vascular endothelial growth factor, collagen) (Table)[1,34,40–42,45–53]; and to accelerate thrombolyisis.[7–16] In general, most of these researchers used a frequency of 1 MHz or 3 MHz, and the intensities ranged from 0.1 to 1.5 W/cm^2. An alternative therapeutic protocol employs a frequency of 45 kHz. An intensity range of 5 to 100 mW/cm^2 was shown to increase the production of IL-1, IL-8, vascular endothelial growth factor, fibroblast growth factor-b, and collagen; promote bone healing; and accelerate thrombolysis.[5,45,54,55] The long-wave (45-kHz) ultrasound increases penetration depth and, therefore, seems to be more appropriate than traditional high-frequency ultrasound (1 MHz and 3 MHz) for promoting revascularization and bone healing at greater depths.

Ultrasound, Signal Transduction, and Gene Regulation

Additional reports suggest that ultrasound alters cellular membrane properties (cellular adhesion, membrane permeability, calcium flux, and proliferation), possibly activating signal-transduction pathways that lead to gene regulation (Table).[37,39–42,45,48,53,56,57] Importantly, exposure to ultrasound caused an increase in intracellular calcium in fibroblasts, suggesting that the mechanical effects disrupt the normal function of the membrane, permitting leaking of calcium into the cell.[49] After ultrasound exposure, the cells rapidly expelled the calcium and returned to a homeostatic state. Mortimer and Dyson[49] eliminated the effects of transient cavitation and gross heating as possible mechanisms for the resultant increases in intracellular calcium. Cells employ calcium as a cofactor in regulating the activity of enzymes, many of which are associated with signal-transduction pathways. Activation of calcium-sensitive signal-transduction pathways (protein kinase C and cyclic AMP) commonly results in gene activation. The resultant protein production could modulate intracellular functions and the activity of surrounding cells.[58–60] A number of the experiments reviewed in the Table demonstrated increases in specific proteins after exposure of cells to therapeutic levels of ultrasound. Combined, these findings suggest that therapeutic ultrasound can modulate signal-transduction pathways that lead to gene regulation or the modulation of RNA translation to a protein product, or both.

Frequency Resonance Hypothesis

Cumulatively, the data may suggest that the mechanical energy within the ultrasound wave and the shearing force of the wave combine to produce mechanical properties that perturbate the cellular membrane and the molecular structures within the cell. The central premise of the frequency resonance hypothesis is that the mechanical energy within the ultrasound wave is absorbed by proteins, altering the structural conformation of an individual protein or the function of a multimolecular complex. Moreover, the ultrasound wave may induce resonant activity in the protein, modulating the molecule's or multimolecular complex's effector function.

The following discussion employs enzymatic proteins as a molecular model. One can view an enzymatic protein as a physical machine performing a physical function within a cell. Enzymes are commonly found in 1 of 2 conformational shapes: on or off. Movement between these 2 conformations (or 3-dimensional shapes) requires a change in the state of energy, which is normally accomplished by the addition or removal of a phosphate molecule. Once an enzyme within a signal-transduction cascade is activated, the signal is amplified to execute an effector function.

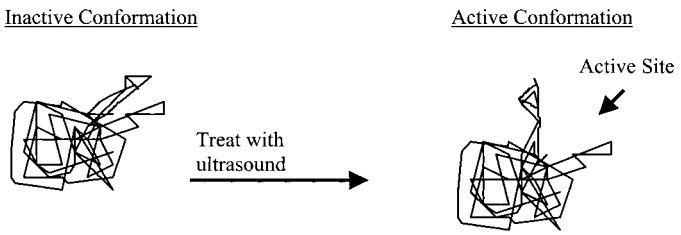

Figure 1. The resonating mechanical force produced by the ultrasound wave may induce transient conformational shifts. Molecules are normally found in one of two 3-dimensional shapes, an "active" or "inactive" conformation.

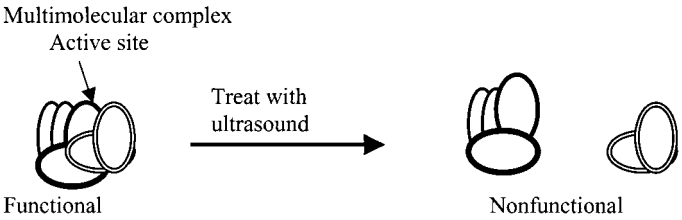

Figure 2. Resonant or shearing forces produced by ultrasound may result in the disjunction of a multimolecular complex with a subsequent loss of function or decrease in activity.

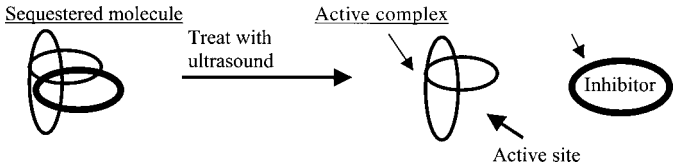

Figure 3. Resonant or shearing mechanical forces produced by ultrasound may dislodge an inhibitor molecule, leading to activation of a signal-transduction pathway.

The frequency resonance hypothesis suggests that the energy provided to the enzyme by the ultrasound wave may induce transient conformational shifts in certain enzymatic proteins, altering the enzyme's activity (ie, kinases or phosphatases) and the overall function of the cell (Figure 1). Alternatively, ultrasound's resonating force may result in the dissociation of functional multimolecular complexes (Figure 2) or the release of a sequestered molecule by dislodging an inhibitor molecule from the multimolecular complex (Figure 3). In essence, the mechanism of ultrasound's action in Figures 2 and 3 is the same. Ultrasound disrupts a multimolecular complex. However, Figure 2 represents a functionally active complex, while Figure 3 represents a functionally sequestered molecule. One can view an inhibitor molecule as a ''safety block'' that functionally inhibits or sequesters a protein from working. When the safety block is released, the protein is then operable.

Shearing forces produced by ultrasound may also play a role in the dissociation of multimolecular complexes. Hypothetically, frequency resonance may imply that different frequencies (1 MHz, 3 MHz, 45 kHz, and others) establish unique resonant or shearing forces (or both). Moreover, various frequencies may affect combinations of proteins or multimolecular complexes in different ways, lending to the possibility of targeted effects at the cellular and molecular levels.

The frequency resonance hypothesis differs from acoustic streaming and cavitation at the basic levels. First, acoustic streaming relates to the movement of objects from one place to another as a function of the force of the wave. In terms of ultrasound therapy, phonophoresis is commonly used to move medication transdermally. Second, cavitation relates to the oscillation of microscopic gas bubbles that may, in turn, affect the cell or cellular process. However, the frequency resonance hypothesis relates to the absorption of ultrasound by proteins and protein complexes that may directly result in alterations to signaling mechanisms within the cell, either by inducing a conformational shift or by disrupting a multimolecular complex.

In the original experiment investigating whether ultrasound could alter protein activity, the researchers reported no effect with respect to the monomer or dimer form of the enzyme creatine kinase.[61] However, Chetverikova et al[61] reported that ultrasound decreased the activity of the dimeric and tetrameric forms of creatine kinase and suggested that the decrease in activity was due to the disruption of the multimolecular forms of creatine kinase (represented in Figure 2). The authors inferred that ultrasound did not directly affect enzyme activity and that the primary acousto-biological interaction appeared to be occurring at a higher level of organization complexity. However, more recent investigations[7–16,54,55] have shown that ultrasound increases thrombolysis, demonstrating that ultrasound can increase enzyme activity (represented in Figure 1). These data support the tried research saying, ''The absence of proof is not the proof of absence.''

The concept of the absorption of ultrasonic energy by enzymatic proteins leading to changes in the enzymes' activity is not novel[61,62]; however, the demonstration that ultrasound can increase or decrease protein activity and possible gene regulation is more recent.[7–16,54–56] While considerable data exist, a model suggesting a molecular mechanism for how the absorption of ultrasound by proteins affects the cell function is novel and is presented here for the first time.

Frequency resonance is one possible explanation of why exposure to ultrasound increased enzymatic activity, resulted in thrombolysis, and did not alter the activity of the enzymes creatine kinase, lactate dehydrogenase, hexokinase, and pyruvate kinase.[7–16,54,55,61] A simple analogy would be 2 tuning forks located at one end of a room, with different frequencies A and B. At the opposite end of the room is a third tuning fork with the same frequency as fork A. Fork A is struck, generating a sound. The sound waves travel through the air and are absorbed at the other end of the room by fork A to produce the same resonating sound, while fork B remains silent. The possibility exists that thrombolysis is affected by the frequencies ''designated'' as therapeutic, while creatine kinase, lactate dehydrogenase, hexokinase, and pyruvate kinase are not.[7–16,54,55,61] Importantly, these reports do not directly demonstrate that a conformational shift is occurring in these enzymes but support the hypothesis.

On the surface, it may appear that the observed decrease in creatine kinase activity and an increase in thrombolytic activity are contradictory; however, a fundamental difference exists. The decrease in activity of the creatine kinase was, most likely, a result of the disruption of the multimolecular dimeric or tetrameric (or both) forms of creatine kinase (Figure 2), while the increased thrombolytic activity may be more associated with activation through harmonic resonance (Figure 1).

In any case, the mechanical effects of ultrasound may result in either the activation or inactivation of an enzymatic protein or a dissociation of a protein complex, leading to alterations in

signal transduction. The frequency resonance hypothesis may describe the molecular mechanism or mechanisms responsible for alterations in cellular membrane properties,[34,39,41,46,49] increases in protein production,* and modulation of enzyme activity.[7–16,54–56]

Frequency resonance and shearing forces on multimolecular complexes may combine to produce the nonthermal effects of therapeutic ultrasound. Collectively, the experiments reviewed here support the frequency resonance hypothesis and demonstrate that therapeutic ultrasound may modulate signal-transduction pathways and gene products associated with the inflammatory response and cells directly involved in the healing response (see Table).

Clinical Implication of Ultrasound Research at the Cellular and Molecular Levels

The purpose of this paper is to raise the awareness that therapeutic levels of ultrasound (1 MHz, 3 MHz, and 45 kHz) stimulate cellular and molecular effects within cells that are centrally involved in the inflammatory and healing processes (Table).[1,5,7–16,34,40–42,45–55] Cumulatively, these reports provide important information that may lead to a better understanding and clinical application of therapeutic ultrasound.[1,5,7–16,34,40–42,45–55] Currently, no clear guidelines exist that provide the clinician with protocols directing when in the injury and healing response ultrasound should be administered, nor are there guidelines on the frequency, intensity, treatment times, or number of treatments required for efficacy. In a recent review of the literature, a wide spectrum of ultrasound treatment protocols was found.[63] In the 10 papers found to be scientifically acceptable, a broad range of treatment settings and methods was used, including (1) nine different clinical indications, (2) five different frequencies, (3) continuous and pulsed output, (4) W/cm^2 ranging from 0.02 to 2.6, (5) treatment time ranging from 2 to 15 minutes, and (6) energy density ranging from 2 to 150 J/cm^2.[64–70] Due to the variety of clinical indicators and methods employed,[63–70] sufficient clinical data are currently not available to generate scientifically sound recommendations for treatment protocols.

The ultrasound treatment protocols employed to investigate the cellular and molecular effects (see Table) were usually a 10-minute exposure at 1 or 3 MHz and 0.1 to 1.5 W/cm^2.[5,39–42,45,48,50,53,56] The experimental protocol investigating thrombolysis ranged from 10 to 200 minutes of exposure at 0.17 to 3.4 MHz and 0.25 to 8.0 W/cm^2, both continuous and intermittent.[7–16] A third experimental protocol employing a frequency of 45 kHz and an intensity range of 5 to 100 mW/cm^2 resulted in increased production of IL-1, IL-8, vascular endothelial growth factor, fibrinoblast growth factor-b, and collagen, promotion of bone healing, and acceleration of thrombolysis.[5,45,54,55]

The logical argument is that in vitro effects cannot be directly applied to clinical treatment protocols. However, from a cellular biology point of view, a strong argument can be made that if a stimulus reaches a critical threshold (eg, consider depolarization thresholds) the cell will respond, regardless of whether the cell is in vitro or in vivo (eg, insulin, histamine, aspirin, or any of the proteins listed in the Table). Importantly, cells in tissue culture usually respond to nanogram or microgram per milliliter quantities of a stimulus. However due to pharmocokinetics (ie, administration, absorption, distribution, and elimination of a drug), higher concentrations and multiple doses per day are normally required to achieve clinical efficacy.

While general recommendations for ultrasound treatment suggest 5 to 10 minutes of exposure and 1 to 3 treatments per day, clinical treatments are almost exclusively done once a day. The possibility exists that clinical treatment protocols commonly employed for ultrasound are not sufficient for therapeutic efficacy. In the review by Robertson and Baker,[63] both of the methodologically acceptable studies showing clinical efficacy used pulsed ultrasound (1:4) with treatment times of 15 minutes, resulting in energy densities of 60 and 150 J/cm^2, respectively.[64,65] Conversely, the remaining 5 studies lacking efficacy employed pulsed ultrasound and exposure times of 2 to 10 minutes, resulting in overall energy densities of 2 to 40 J/cm^2.[66–70] The frequency resonance hypothesis may suggest that different ultrasonic frequencies (1 MHz, 3 MHz, 45 kHz, and other) may require different durations of exposure (time), different energy densities (J/cm^2), or both, to reach therapeutic efficacy.

The identification and scientific understanding of therapeutic ultrasound's nonthermal mechanisms may lead to a comprehensive and effective clinical strategy. Further research is needed on 2 fronts: (1) cellular and molecular research to determine whether the mechanical mechanisms proposed by the frequency resonance hypothesis can be elucidated and provide insight into a comprehensive strategy for the clinical indications of therapeutic ultrasound at various frequencies, and (2) methodologically sound clinical research designed to provide meaningful input and outcome measures related to clinical efficacy. Both avenues of research should strive to establish time and dose-dependent response curves.

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