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Cellular Signaling Pathways Modulated by Low-intensity Extracorporeal Shock Wave Therapy

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Abstract

Low-intensity extracorporeal shock wave therapy (Li-ESWT) is a form of energy transfer that is of lower intensity (<0.2 mJ/mm^2) relative to traditional Extracorporeal Shock Wave Lithotripsy (ESWL) used for management of urinary stones. At this intensity and at appropriate dosing energy transfer is thought to induce beneficial effects in human tissues. The proposed therapeutic mechanisms of action for Li-ESWT include neovascularization, tissue regeneration, and reduction of inflammation. These effects are thought to be mediated by enhanced expression of vascular endothelial growth factor, endothelial nitric oxide synthase, and proliferating cell nuclear antigen. Upregulation of chemoattractant factors and recruitment/activation of stem/progenitor cells may also play a role. Li-ESWT has been studied for management of musculoskeletal disease, ischemic cardiovascular disorders, Peyronie's Disease, and more recently erectile dysfunction (ED). The underlying mechanism of Li-ESWT for treatment of ED is incompletely understood. We summarize the current evidence basis by which Li-ESWT is thought to enhance penile hemodynamics with an intention of outlining the fundamental mechanisms by which this therapy may help manage ED.

Introduction

Shock waves may be conceptualized as acoustic waves that propagate through a medium (such as human tissues) and carry energy [1]. The angle of the waves generated by a shock wave device may be adjusted so that the energy they carry converges on a single point in space. [2] While the energy of each individual wave is low, when brought to a point of confluence the summation of each individual wave's energy may produce an effect on a target. Hence, it is possible to transmit energy to a remote anatomical target with minimal effect on the tissue located between the shockwave generator and the target. This principle is the foundation of Extracorporeal Shockwave Lithotripsy (ESWL) used to fragment renal and ureteral stones.

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The use of ESWL requires transfer of a substantial amount of energy in order to fracture a stone. Although the principle medical utilization of external shock wave energy has historically been towards fracture of stones, a counterintuitive osteoblastic response was observed in animal studies [3]. This work led to clinical application of Low-intensity extracorporeal shock wave therapy (Li-ESWT) to numerous orthopedic disorders,[4] including healing of fractures, [5] pain management, and treatment of arthritis[6].

Shock waves may also be applied to soft tissues, wherein they may induce a cascade of biological reactions. The energy transfer of Li-ESWT occurs via mechanotransduction[7], micro-cavitation[8], and thermodynamic effects. The dominant effect of low-intensity shock waves is thought to be mechanotransduction. Rather than inducing damage in the target, Li-ESWT may induce tissue healing and angiogenesis[9]. This effect has led to application of Li-ESWT for treatment of muscular disorders[10], cardiac disease[11],[12], non-healing wounds[13],[14], and Peyronie's Disease (PD) [15]. [16]

Unfortunately, Li-ESWT has not shown reliable and clinically relevant changes in PD [17]. Despite this disappointing result, sexual medicine specialists have remained interested in this technology as a means to manage erectile dysfunction (ED)[18], [19]. Promising clinical results in the initial study of Vardi et al[20] and subsequent studies from other centers have generated marked interest in this novel ED treatment [21]. Despite this high level of interest, the underlying mechanism of action for Li-ESWT in management of ED remains unclear. Existing studies suggest that the effects of Li-ESWT on penile tissue include cell proliferation, enhanced cell survival, mitigation of fibrosis/inflammation, and recruitment/activation of endogenous stem cells [22]. The net result may be angiogenesis, improved wound healing, and regeneration of muscle and nerve tissue.[23-25]

Li-ESWT regulates the cellular signaling transduction, affects the transcription and modification of intracellular proteins.[24] Specific cellular processes/molecules modulated by Li-ESWT include Focal Adhesion Kinase (FAK)[3], Extracellular-signal-regulated kinase (ERK)[23], Wnt[26], ATP/P2X7[26], Protein kinase R-like endoplasmic reticulum kinase / activated transcription factor (PERK/ATF)[27],Vascular Endothelial Growth Factor (VEGF) and Brain-derived neurotrophic factor (BDNF)[28]. We will elaborate on these molecular mechanisms in the following (Figure 1).

Characteristics of medical shockwaves

Shock waves are characterized by high peak pressures (up to 100 mpa or higher), rapid pressure rise (<10 ns), short duration (<10 ms) and wide frequency range[7]. Unlike ultrasonic waves (which consist of periodic oscillations with limited bandwidth), shock waves consist of a single predominantly positive pressure pulse followed by a relatively small stretched wave component[2]. Shock waves used for biomedical purposes are generated in a fluid medium using an electro-hydraulic, piezoelectric, or electromagnetic generator. [29] Generated shockwaves are then directed to the target by a focusing unit. [2] It is unclear on whether the method of shock wave generation is germane to ultimate tissue effects; however, the intensity of the generated wave is likely to be highly relevant.

Shock waves and Mechanotransduction

Li-ESWT exerts a mechanical force on cell membranes and contents. Mechanotransduction is the term for cellular processes by which mechanical stimuli are converted into biochemical signals. Short-term reactions include increased intracellular tension, cellular adhesion, and cellular migration. Long-term effects of Li-ESWT are thought to be mediated through multiple overlapping and crosstalk signaling pathways (e.g. protein synthesis/ secretion, structural reorganization, proliferation, vitality)[30].

Mechanotransduction consists of several discrete phases: [31]

Phase 1: the mechanocoupling phase, wherein wave energy is converted into a mechanical signal in the vicinity of the cell;

Phase 2: biochemical coupling, wherein the mechanical signal induces activation of biochemical pathways, leading to activation of transcription factors and/or changes in the action of cellular pathways such as calcium-dependent pathways, mitogen-activated protein kinases, and second messenger systems;

Phase 3: signal transmission, where the biochemical signal may be propagated between cells;

Phase 4: cellular response.

A number of specific elements are thought to play a role in mechanotransduction. These include: [7, 30]

1) **Extracellular matrix proteins** (e.g fibronectin), which can transmit mechanical forces to cells;

2) **Stretch-activated ion channels**, which allow influx of calcium and other ions with membrane strain;

3) **The glycocalyx,** which responds to fluid shear stress on the cell membrane of endothelial cells and mediates endothelial permeability and action;

4) **Cell-cell junctional receptors and cell-matrix focal adhesions,** which may induce paracrine or ionic effects;

5) **Cytoskeletal components,** which may altering binding affinity and/or concentration for a particular molecule/signaling pathway;

6) **The nucleus,** the membrane of which is known to contain mechanosensors;

7) **The cytoplasm,** compression of which by a mechanical force may alter the effective concentration of autocrine and paracrine signaling molecules such as caveloae and membranes of organelles;

8) **The mitochondria,** which are affected by shock waves that induce changes in ATP production.

Cellular membranous signaling pathways modulated by Li-ESWT

a). Focal Adhesion Kinase (FAK) cellular signaling pathway

Integrin is a transmembrane receptor that facilitates cell-extracellular matrix (ECM) adhesion and also plays a role conducting signals from the extracellular matrix into the cytoplasm [32] Integrin activates signal transduction pathways that regulate the cell cycle, organization of the intracellular cytoskeleton, and movement of new receptors to the cell membrane. Focal Adhesion Kinase (FAK) is a non-receptor cytosolic protein tyrosine kinase (PTK) with a central catalytic domain flanked by large N- and C-terminal domains. FAK indirectly localizes to sites of integrin-receptor clustering through interactions with integrin-associated proteins[33]. FAK is a central mediator of integrin signaling as well as an important part of several other signal pathways including those that influence cell growth, differentiation, and apoptosis [34].

Low-energy shock waves interact with integrin and activate FAK by phosphorylation mediated by integrin α5 and β1, thereby triggering a series of cellular signaling and related biological changes which are known to be relevant to osteoblast adhesion and migration[3]. One of these downstream effects is phosphorylation of Tyrosine 397, the major autophosphorylation site of FAK; this response is known to play a role in cell migration[35, 36]. Additional Li-ESWT-mediated effects on FAK include translocation to focal adhesions and enhancement of phosphorylation of paxillin. Mechanical forces also induce focal adhesion maturation and α-actin redistribution to focal cellular adhesions.

The mammalian target of rapamycin complex 1(mTORC1) is regulated by FAK phosphorylation in the context of Li-ESWT mediated mechanoconduction. Li-ESWT induces mTORC1 phosphorylation and subcellular translocation, which in turn leads to mTORC1 mediated control of cell proliferation[37].

The FAK mediated effects that are thought to be most relevant to management of ED include proliferation and migration of cells that restore endothelial and smooth muscle integrity in penile vascular tissues. The majority of cellular signaling pathways relevant in Li-ESWT modulation of ED appear to work via FAK.

b). Extracellular-signal-regulated kinase (ERK) cellular signaling pathway

The mechanical effects of shock waves on cells may result in changes in membrane activity. This may include opening of Pannexin-1 (PANX1) channel to release ATP[23]. ATP activates p38MAPK and Mek1 / 2-Erk1 / 2 signaling pathway, which lead to cell proliferation in vitro and enhanced wound healing in vivo [23]. The role of ATP in mediating shock wave induced cell proliferation was demonstrated by studies in which ATP was degraded by apyrase or the P2Y receptor blocker suramin; in the presence of these inhibitors, the cellular proliferative effect of shock wave therapy was eliminated. Similar blockade of shock wave induced cell proliferation was demonstrated by inhibition of Mek1 / 2 (via U0126 in vitro and GSK1120212 in vivo) [23].

The Erk 1/2 signaling pathway may also be activated by Li-ESWT via stimulation of the growth factor receptor and a seven-domain G-protein coupled receptor. This cascade activates Ras, which in turn interacts with integrins via the adaptor protein SHC and the guanine nucleotide exchange factor Son of Sevenless (SOS). Integrin-mediated phosphorylation of Erk 1/2 is also associated with enhanced expression of VEGF and increased eNOS expression,[38] the net result of which is endothelial regeneration and angiogenesis through VEGFR2-Akt-eNOS[39]. Given the central importance of vascular integrity to penile erection, restoration of endothelial action is a likely mediator of ESWT-induced improvement in penile erection.

c). Wnt/β-catenin cellular signaling pathway

The Wnt signaling pathway, a highly conserved sequence across taxa, is a complex network of protein action that is relevant for embryonic development, neoplasia, and the normal physiological processes of adult animals[40]. The net effects of the Wnt pathway are both autocrine and paracrine.

Three Wnt signaling pathways have been identified: canonical Wnt pathway, noncanonical Wnt/planar cell polarity pathway (PCP), and noncanonical Wnt/calcium pathway. The Wnt/PCP pathway regulates the cytoskeleton to control cell shape. The noncanonical Wnt/calcium pathway regulates intracellular calcium ion concentration [41]. All three of these Wnt signaling pathways are activated by the binding of Wnt protein ligands to the Frizzled, a G-protein coupled receptor. Frizzled conducts signals to intracellular Dishevelled[42] (Dsh), a cytosolic phosphorylated protein which is involved in both the calcium and non-calcium Wnt signaling pathways[43].

Binding of Wnt to the Frizzled Receptor recruits DVL to the membrane, providing a site for Axin and GSK3β to bind and phosphorylate LRP5 / 6 (transmembrane LDL receptor-associated protein), thereby preventing the constitutive form of β-catenin degradation. After stabilized and accumulated, β-Catenin enter the nucleus and binds to TCF to initiate downstream gene transcription. In the absence of WNT proteins, β-catenin will be phosphorylated on the complex of β-catenin and a tertiary complex formed by axin, APC, CK1α and GSK 3β[44]. β-catenin is phosphorylated by a complex of kinases at a set of conserved Ser and Thr residues at its amino terminus[45]. The phosphorylated form of β-catenin is recognized by E3 ubiquitin ligase (β-TrCP) and then targeted for proteasomal degradation.

One of the potential therapeutic mechanisms of Li-ESWT is smooth muscle proliferation. A number of studies have demonstrated that Li-ESWT enhances activation of Wnt signalin/β-catenin with a peak increase 2 hours post-treatment. Shock wave mediated activation of Wnt-β-catenin has been shown to activate MyoD/Myf5 in vitro, which induces muscle cell differentiation[46].

Shock wave induced activation of Wnt/β-catenin has also been shown to induce differentiation of mouse Neuronal stem cells (NSCs) in vitro[26]. Inhibition of the Wnt/β-catenin signaling pathway by Dkk-1 45 delayed NSC maturation by reducing β-tubulin III

expression in animals treated with Li-ESWT. These findings provide further evidence that Li-ESWT treatment regulates NSCs differentiation via Wnt/β-catenin signaling[47].

Cellular signaling related to Organelle modulated by Li-ESWT

a). Unfolded Protein Response signaling pathway

The endoplasmic reticulum stress (ERS) response is a ubiquitous cellular pathway that directs proteins from the endoplasmic reticulum to the cytosol or nucleus. Protein kinase R-like endoplasmic reticulum kinase (PERK) is one of the major transducers of the ERS response. PERK directly phosphorylates the alpha-subunit of eukaryotic initiation factor 2 (eIF2α), resulting in translational attenuation. Phosphorylated eIF2α specifically promotes translation of activated transcription factor 4 (ATF4), which is known to be an important transcription factor regulating osteoblast differentiation and bone formation. Mechanical forces are known to activate the ERS-mediated PERK-eIF2α-ATF4 signaling pathway and thus modulate the differentiation of osteoblasts[48]. In addition to ERS-mediated effects on bone metabolism, Li-ESWT[27] increases myotube formation in muscle-derived stem cells (MDSCs) via the PERK pathway. This process of myogenesis is thought to play a role in regeneration of penile smooth muscle and subsequent improvement in penile erection.

Li-ESWT activates the protein kinase RNA-like endoplasmic reticulum (ER) kinase (PERK) pathway by increasing the phosphorylation levels of PERK, eukaryotic initiation factor 2a (eIF2α), and activating transcription factor 4 (ATF4) in an energy-dependent manner. GSK2656157—an inhibitor of PERK—effectively blocks the effect of Li-ESWT on the phosphorylation of PERK, eIF2α, and the expression of ATF4. Furthermore, silencing ATF4 dramatically attenuates the effect of Li-ESWT on the expression of BDNF but has no effect on hypoxia-inducible factor (HIF)1α or glial cell-derived neurotrophic factor (GDNF) in Schwann cells. It is apparent that Li-ESWT stimulates the expression of BDNF through activation of PERK/ATF4 signaling pathway[49]. (Fig 2)

b). ATP/P2X7 pathway

Activation of the ATP/P2X7 pathway may also be of benefit in treatment of ED. Osteoblasts express several P2 receptor subtypes, ATP can activate P2 receptors in osteoblasts and increases intracellular Ca2+ concentrations. P2 signaling also regulates the activity of ERK1/2 and P38/MAPK signaling pathways and c-fos expression in osteoblasts. Stimulation of the P2X7 receptor activation can lead to osteoblast differentiation and enhancement of cell function for bone formation and remodeling.

The P2X7 receptor is the most abundantly expressed ionic subtype, and there is increasing evidence that human osteoblasts express the P2X7 receptor. Based on this evidence, some researchers have confirmed that shock waves may lead to the release of ATP and activation of P2X7 receptors. In addition, ATP can activate p38/MAPK, which plays an important role in regulating the differentiation of human bone marrow mesenchymal stem cells into osteoblasts. P38/MAPK activation has also been shown to induce the expression of c-fos and c-Jun[50]. In this study shockwave treatment did not significantly inhibit cell survival

(viability > 95%) if the dose was restricted to 200 or fewer pulses. Cell survival declines in a dose dependent fashion as the number of shocks exceeds 200[51].

Cellular signaling related to Growth factor modulated by Li-ESWT: *BDNF cellular signaling pathway*

BDNF supports the survival of neurons and promotes the growth and differentiation of new neurons. BDNF has two major receptors: the pan-NT receptor p75 (p75NTR) and the tropomyosin-associated receptor kinase B (TrkB). P75 NTR has many functions such as differentiation, cell survival, and cell death signals. Binding of BDNF to TrkB triggers activation of downstream signaling pathways such as RAS/MAPK, RAP/MAPK, PI3K/AK, and phosphoinositide phospholipase C (PLC gamma) pathways. These pathways lead to cell survival, differentiation, synapse formation and plasticity [52].

Expression of BDNF increases markedly 3 days after nerve injury. After that, the expression of BDNF decreases sharply and reaches basal level 10 days after the injury. Treatment with repeated Li-ESWT increased expression of BDNF in the penis at a level that was maintained up to 26 days after nerve injury[49]. This prolonged expression is likely to sustain neurotrophic healing beyond what can be expected naturally and without intervention.

The mechanism of Li-ESWT mediated BDNF activation was studied in RT4-D6P2T Schwann cells and found to be related to activation of PERK/ATF4. The PERK blocker GSK2656157[53] reduced Li-ESWT mediated eIF2α phosphorylation and downstream target gene ATF4 expression. In this context expression of BDNF was also significantly reduced[49].

Other studies show that Li-ESWT induces MSCs to express VEGF, though the underling mechanism is not well understood. VEGF in turn upregulates the PI3K / AKT /mTOR pathway, which induces autophagy. Autophagy and apoptosis assays indicate that Li-ESWT activates autophagy in penile tissue and effectively reduces apoptosis. BDNF is also associated with VEGF expression[54]. Knockdown of BDNF significantly reduces VEGF expression and angiogenesis in vivo[55]. There are data supporting the contention that Li-ESWT mediated increases in BDNF may also enhance VEGF expression in MSCs. Similar effects (BDNF mediated enhancement of VEGF signaling) have been reported in MSC treated with Li-ESWT.[56].

Perspectives

Li-ESWT is a non-invasive and promising physical method for regenerative medicine. Numerous animal models of erectile dysfunction indicate that Li-ESWT may improve angiogenesis, activation of stem/progenitor cells, and muscle/nerve regeneration for remodeling penile erectile tissues. A robust and growing body of evidence supports the utility of Li-ESWT in ED; involved pathways include FAK[3], ERK[23], Wnt[26], ATP/P2X7[26], PERK/ATF[27], and BDNF[28]. Further research will be required to better define the key pathways relevant to erection function recovery and optimization of treatment protocols.

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Abbreviation

ATF	activated transcription factor
BDNF	Brain-derived neurotrophic factor
Dsh	Dishevelled
ECM	cell-extracellular matrix
ED	erectile dysfunction
eIF2	eukaryotic initiation factor 2
ERK	Extracellular-signal-regulated kinase
ERS	endoplasmic reticulum stress
ESWL	Extracorporeal Shock Wave Lithotripsy
FAK	Focal Adhesion Kinase
GDNF	glial cell-derived neurotrophic factor
HIF	hypoxia-inducible factor
Li-ESWT	Low-intensity extracorporeal shock wave therapy
LRP	LDL receptor-associated protein
MDSCs	muscle-derived stem cells
mTORC1	mammalian target of rapamycin complex 1
NSCs	Neuronal stem cells
p75NTR	pan-NT receptor p75
PANX1	Pannexin-1
PCP	Planar cell polarity pathway
PD	Peyronie’s Disease
PERK	Protein kinase R-like endoplasmic reticulum kinase
PTK	protein tyrosine kinase
SOS	Son of Sevenless

TrkB	tropomyosin-associated receptor kinase B
UPR	Unfolded Protein Response
VEGF	Vascular Endothelial Growth Factor

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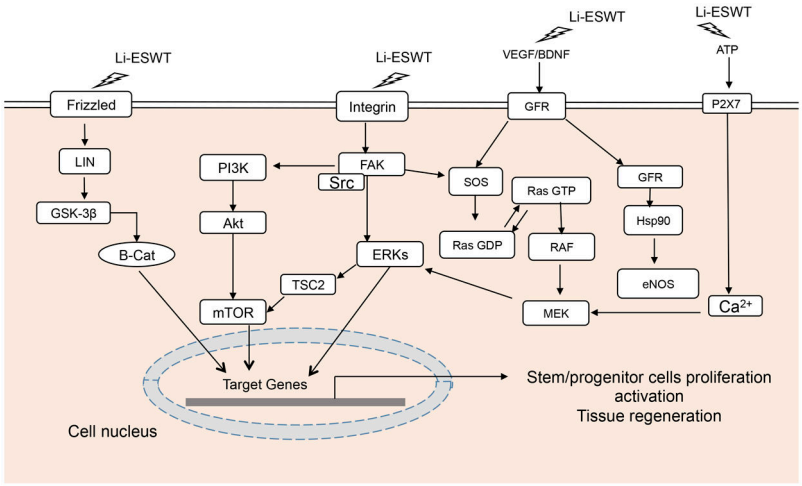

Fig 1. Cellular signaling pathways modulated by Low-intensity Extracorporeal Shock Wave Therapy (Li-ESWT)

Li-ESWT: Low-intensity extracorporeal shock wave therapy; **Frizzled:** receptor of WNT; **BDNF:** Brain-derived neurotrophic factor; **GSK-3β:** Glycogen synthase kinase-3 beta; **β-Cat:** beta-catenin; **PI3K:** Phosphoinositide 3-kinase; **Akt:** Protein kinase B (PKB), also known as Akt; **Src:** Src is short for sarcoma, a Proto-oncogene tyrosine-protein kinase Src; **TSC2:** Tuberous Sclerosis Complex 2; **GFR:** growth factor receptor; RAS: molecules of MAPK/ERK pathway; **RAF:** molecules of MAPK/ERK pathway; **MEK:** mitogen-activated protein kinase kinase; **mTORC1**:mammalian target of rapamycin complex 1; **FAK:** Focal Adhesion Kinase; **VEGF:** Vascular Endothelial Growth Factor ; **SOS:** Son of Sevenless

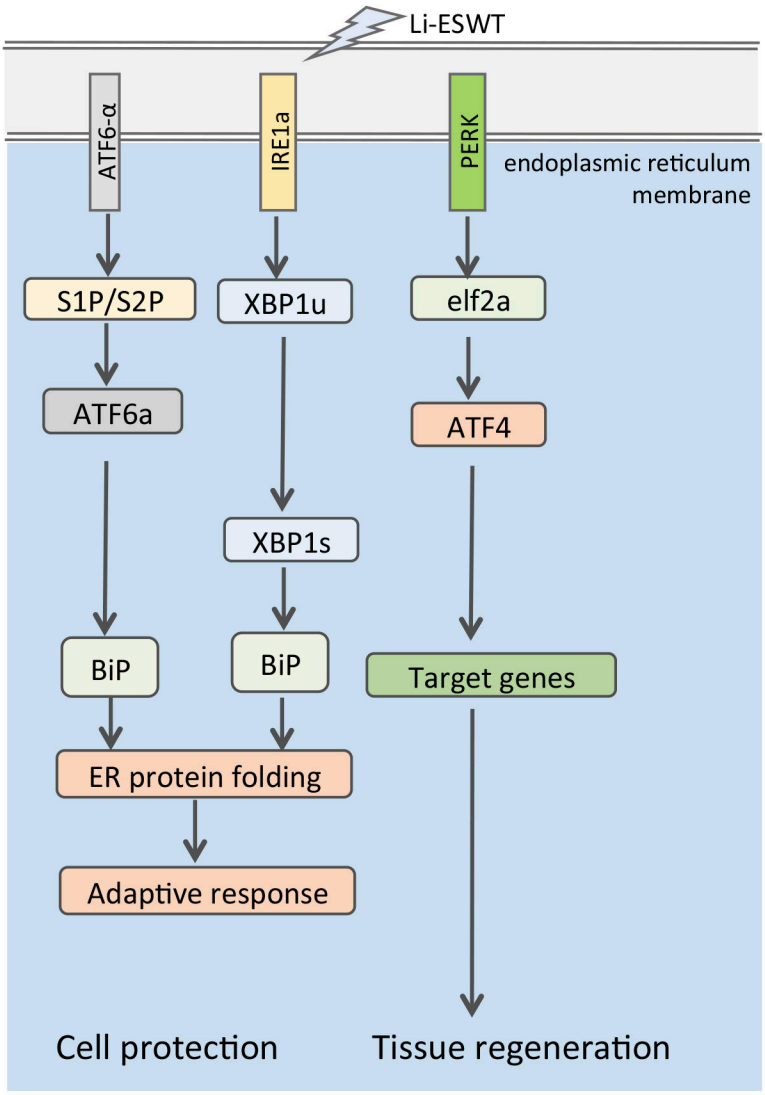

Fig 2. Low-intensity Extracorporeal Shock Wave Therapy Modulated Unfolded Protein Response (UPR)

IRE1a: inositol-requiring enzyme 1α; **S1P:** Sphingosine-1-phosphate; **S2P:** Sphingosine-2-phosphate; **ATF:** activated transcription factor; **BiP:** Immunoglobulin heavy-chain-binding protein; **XBP1u:** un-spliced X-box binding protein 1; **XBP1s:** spliced X-box binding protein 1; **PERK:** Protein kinase R-like endoplasmic reticulum kinase; **eIF2:** eukaryotic initiation factor 2