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UTILIZING ULTRASOUND FOR GENE THERAPY:A PROMISING STRATEGY FOR TISSUE REGENERATION

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ABSTRACT

A promising approach for tissue regeneration, ultrasound-assisted gene therapy offers possibilities for effective and precise gene delivery. This article provides a comprehensive overview of the current state of research and development in ultrasound-assisted gene therapy and its prospective use in tissue regeneration. Gene therapy has potential for treating various diseases and promoting tissue regeneration through introduction of therapeutic genes into target cells. However, the efficient and effective delivery of genes to specific tissues remains a major challenge. Targeted and regulated release of therapeutic genes is made possible by ultrasound-assisted gene therapy, which makes use of the mechanical energy of ultrasonic waves to improve gene delivery. We explore the various techniques and modalities employed, including microbubble-mediated sonoporation, cavitation, and acoustic streaming, and their impact on gene delivery efficiency. Factors influencing gene transfection efficiency, such as ultrasound parameters, microbubble properties, and gene vectors are also considered. Furthermore, the applications of ultrasound-assisted gene therapy in tissue regeneration, including musculoskeletal, cardiovascular, and neural regeneration are also highlighted. We discuss the potential of this approach in enhancing cell proliferation, differentiation, and tissue repair processes. Additionally, we addressed the future challenges in the field, such as optimizing ultrasound parameters, developing safe and efficient gene vectors, and translating ultrasound-assisted gene therapy to clinical settings. Overall, ultrasound-assisted gene therapy shows great promise as a non-invasive and targeted approach for tissue regeneration. With further advancements and translational research, this technology has the possibility to revolutionize the field of regenerative medicine and provide new paths for therapeutic interventions in various diseases.

INTRODUCTION: Tissue regeneration is intricate process that holds tremendous potential for treating various diseases and tissue injuries. Conventional treatment approaches often focus on managing symptoms or replacing damaged tissues with synthetic materials or transplants (1) . However, these methods have limitations and may not fully restore the structure and functioning of the affected organs or tissues. As a result, researchers have been exploring innovative strategies to enhance tissue regeneration, and one such promising approach is ultrasound-assisted gene therapy. "Gene therapy deals with the introduction of therapeutic genes into target cells to correct genetic abnormalities, promote tissue repair, this leads to enhanced therapeutic outcomes (2) . It offers a potential solution for addressing the underlying causes of diseases rather than just managing the symptoms. However, efficient gene delivery to the desired tissue remains a major challenge in gene therapy. Conventional gene delivery methods, such as viral vectors or physical techniques like electroporation, often suffer from limitations related to targeted specificity, efficiency, and safety (3) . Ultrasound-assisted gene therapy has become a novel and powerful technique to overcome these limitations and improve gene delivery efficiently. Ultrasound waves, consisting of high-frequency sound waves beyond the audible range, can penetrate tissues non-invasively and generate mechanical forces that can enhance gene transfer (4) . By coupling ultrasound waves with gene vectors and enhancing the permeability of target cells, gene therapy with help of ultrasound enables precise and targeted delivery of therapeutic genes to specific tissues. The use of ultrasound in gene therapy offers several advantages. Firstly, ultrasound waves can be precisely focused, allowing for spatially controlled gene delivery. This feature is particularly beneficial when targeting deep-seated tissues or specific regions within an organ. Secondly, ultrasound is non-invasive and safe, making it an attractive alternative to more invasive delivery methods. Additionally, ultrasound can be readily integrated with imaging techniques, allowing real-time monitoring and guidance during gene delivery procedures (5) .In this review article, a comprehensive overview of ultrasound-assisted gene therapy as a promising approach for tissue regeneration. We discuss the underlying principles and mechanisms of ultrasound-assisted gene delivery, including microbubble-mediated sonoporation, cavitation, and acoustic streaming. We explore the impact of various ultrasound parameters, such as frequency, intensity, and duration, on gene transfection efficiency (6) . Moreover, we examine the properties of microbubbles and gene vectors and their influence on ultrasound-mediated gene delivery. Furthermore, we delve into the applications of ultrasound-assisted gene therapy in tissue regeneration, including its potential in musculoskeletal, cardiovascular, and neural regeneration (9) .We highlight studies demonstrating the enhancement of cell proliferation, differentiation, and tissue repair processes using ultrasound-assisted gene therapy. We also address the new challenges and look for future endeavors in the field, such as optimizing ultrasound parameters, developing safer and more efficient gene vectors, and translating ultrasound-assisted gene therapy to clinical settings. In conclusion, ultrasound-assisted gene therapy represents a promising approach for tissue regeneration, offering targeted and efficient gene delivery to enhance tissue repair and therapeutic outcomes (7).

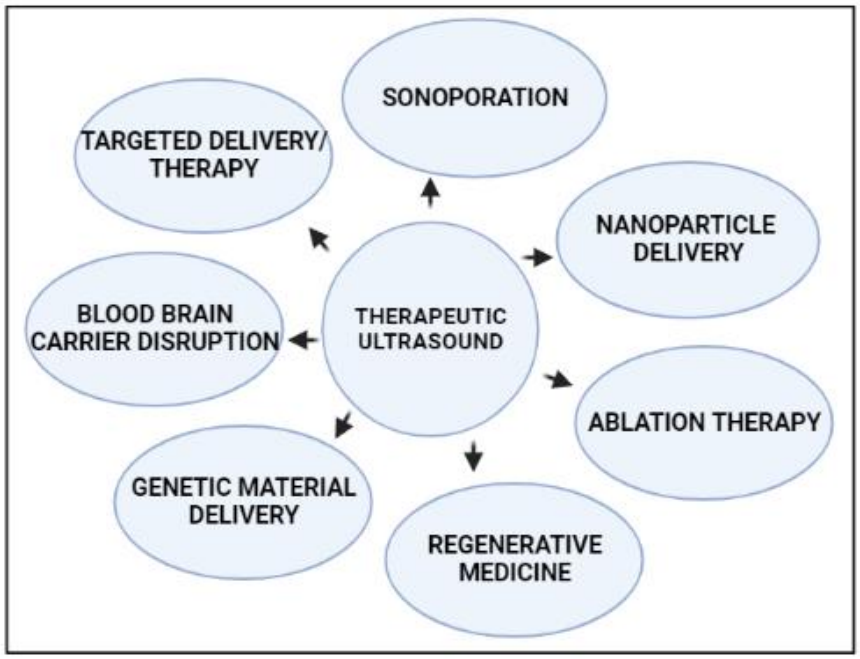

Figure1. Applications of the ultrasonic treatment

With further advancements in ultrasound technology, gene vectors, and translation into clinical practice, this innovative technique can revolutionize the field of regenerative medicine and guide us with new approaches for treating various diseases and injuries (8) . Created with BioRender.com

Table 1 provides a comparison of the benefits and drawbacks associated with *Delivery mechanism involving the use of viruses* and *Delivery mechanism without the use of viruses.*

Method of delivering genes	Benefits	Drawbacks	Sources
Delivery mechanism involving the use of viruses	achieves a high rate of successful gene delivery and expression in the targeted cells	Suboptimal efficacy Restricted gene size, limited to a single copy Concerns regarding stability and regulation Potential for viral infection risk Immunogenicity and toxicity issues High cost and prolonged procedure Restricted applicability to specific tissues and cells	(10)
Delivery mechanism without the use of viruses	Absence of infection Minimal toxicity No size restrictions on the gene Cost-effective and straightforward to prepare Long-lasting stability, suitable for all types of tissues and cells	Inadequate transfection Efficiency	(1)

2. Delivery of genes through sonoporation

Gene delivery through sonoporation is considered as a promising approach for tissue regeneration. Ultrasound-assisted gene therapy offers a non-invasive and targeted method to introduce therapeutic genes into specific tissues, enabling precise control over gene expression and promoting tissue repair (12) . Our review article discuss the recent advancements and potential applications of ultrasound-assisted gene therapy in tissue regeneration. One of the key advantages of sonoporation-based gene delivery is its ability to enhance gene transfer efficiency (14) . Ultrasound waves in combination with microbubbles create transient pores in the cell membrane, allowing for the efficient uptake of therapeutic genes. This technique has been successfully employed in various tissues, including skeletal, cardiac, and peripheral tissues, to promote tissue regeneration and repair (13) . In skeletal tissue regeneration, sonoporation-mediated gene delivery has shown promising results. Researches have demonstrated the successful delivery of genes encoding growth factors, such as bone morphogenetic proteins (BMPs), to stimulate osteogenesis and enhance bone formation (15) . The use of ultrasound in conjunction with specific gene constructs has shown improved bone healing outcomes in preclinical models, offering a potential therapeutic strategy for bone-related disorders and fractures. In cardiac tissue regeneration, sonoporation-based gene therapy has shown great potential for the treatment of myocardial ischemia (16) . Delivering of genes encoding angiogenic factors, like vascular endothelial growth factor (VEGF), through sonoporation has been shown to enhance neovascularization and improve blood flow in ischemic myocardium. This approach holds promise for the management of myocardial infarction and ischemic heart diseases, offering an alternative or complementary strategy to conventional therapies. Furthermore, sonoporation has been explored as a means to treat peripheral ischemia. By delivering genes encoding angiogenic factors to the ischemic tissues, such as the hindlimbs, sonoporation can stimulate angiogenesis and improve blood flow, leading to tissue reperfusion and functional recovery. Studies have demonstrated the efficacy of this approach in preclinical models, paving the way for potential clinical translation in the management of peripheral arterial diseases. Despite the significant progress in the field of ultrasound-assisted gene therapy, several challenges need to be addressed for successful clinical translation (10) . Optimization of ultrasound parameters, such as frequency, intensity, and exposure time, is essential to ensure efficient and safe gene delivery. Additionally, the development of targeted gene delivery strategies, such as ligand-conjugated microbubbles, can further enhance tissue specificity and improve therapeutic outcomes. In conclusion, sonoporation-mediated gene delivery holds great promise for tissue regeneration (17) . This non-invasive and targeted approach offers a versatile platform for the delivery of therapeutic genes to various tissues, including skeletal, cardiac, and peripheral tissues. With further advancements in ultrasound technology, optimization of gene constructs, and understanding of the underlying mechanisms, ultrasound-assisted gene therapy has the potential to revolutionize tissue regeneration strategies and cleared the path for new interventions in the field of regenerative medicine. However, sonoporation also has several limitations. The efficiency of gene delivery can vary, depending on the ultrasound parameters used and the properties of the cells being targeted. In addition, the process can be associated with toxicity, and careful optimization of the parameters is required to minimize these effects. Nonetheless, sonoporation is a promising method for gene delivery and is being actively investigated in both research and clinical settings.

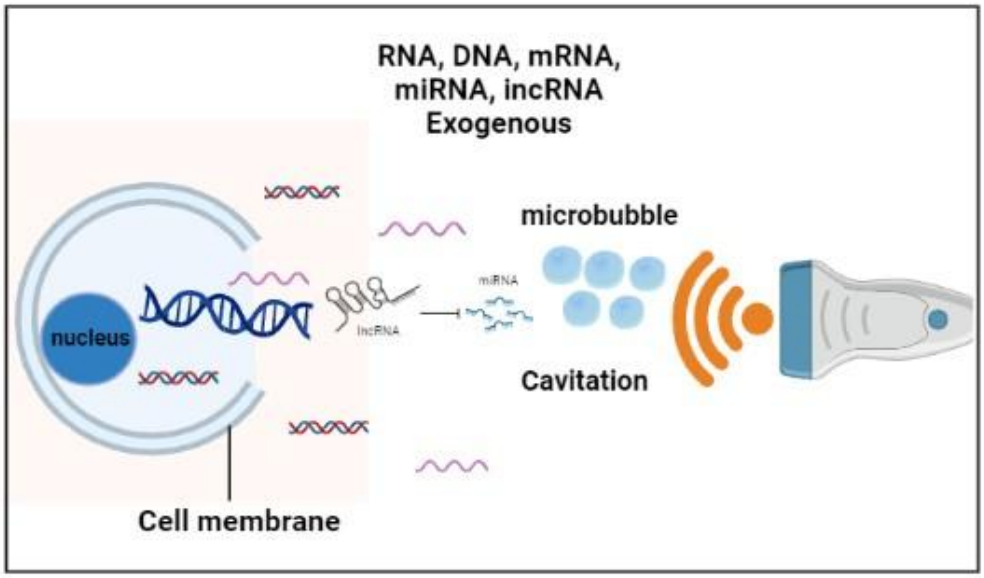

Figure 2 depicts the mechanism by which microbubble cavitation induces the formation of a pore in the cellular membrane, facilitating the passive diffusion of exogenous nucleic acids into the cytoplasm. Created with BioRender.com

Moreover, the choice of microbubbles employed can impact cell viability and the subsequent expression of genes. Limited direct comparisons have been made between biotinylated cationic microbubbles and their neutral counterparts; however, the former has demonstrated superiority in terms of enhanced attachment to cells and nucleic acids (18). Additionally, these biotinylated cationic microbubbles have shown a protective effect when the ultrasonic power density is increased. Ongoing investigations are focusing on cell viability and gene expression (19). The selection of plasmids in experimental studies has been observed to impact the duration of gene expression, with attempts made to extend expression in vivo for as long as 85 days. Previous research has suggested that incorporating genes into an implanted scaffold or matrix can significantly enhance gene transfection induced by sonoporation in the specific area, potentially increasing it by up to 25 times. Additionally, the inclusion of a scaffold can offer protection to implanted stem cells, the target population for gene transfection, by attracting endogenous progenitor/stem cells to the site of injury (20). However, it is important to address the potential issue of ultrasonic wave attenuation caused by implanted materials, which requires further investigation.

3. Utilizing Sonoporation in Tissue Regeneration

The delivery of genes or genetic materials to cells is a prominent application of sonoporation in the context of tissue regeneration. Ultrasound waves are used to create small openings in the cell membrane, genetic material can be introduced into cells, modifying their behavior and promoting tissue regeneration (21). This approach has been extensively explored in different contexts, such as bone, muscle, and skin regeneration, where sonoporation has demonstrated its efficacy in delivering genes encoding bone morphogenetic proteins (BMPs). These proteins serve as essential signaling molecules involved in the promotion of bone growth and facilitating repair processes. Studies have shown that sonoporation-mediated delivery of BMP genes to bone cells can promote bone regeneration in animal models of bone injury and diseases. In muscle regeneration, sonoporation is used to deliver genes encoding myogenic transcription factors, which are proteins that regulate the development and repair of muscle tissue (22). Studies have shown that the sonoporation-mediated delivery of these genes can promote muscle regeneration in animal models of muscle injury and disease. Sonoporation has been used to deliver genes encoding growth factors and extracellular matrix proteins, which are molecules that play a critical role in skin repair and regeneration. Studies have shown that the sonoporation-mediated delivery of these genes can promote wound healing and skin regeneration in animal models of skin injury and disease (23).

Another approach to sonoporation in tissue regeneration is to enhance the delivery of growth factors or other therapeutic agents to cells and tissues. Growth factors are signaling molecules that

play a vital role in tissue regeneration by promoting cell proliferation, differentiation, and migration phenomenon (24) . By using sonoporation to enhance the delivery of growth factors to cells and tissues, the regeneration of damaged or injured tissues can be accelerated. One illustrative instance of utilizing sonoporation for tissue regeneration is the therapeutic application in the management of chronic wounds. Chronic wounds are a significant medical problem that affects millions of people worldwide and are difficult to treat due to impaired healing processes. It is possible to enhance the healing process and promote tissue regeneration by using sonoporation to deliver growth factors to the wound site (25) . In conclusion, sonoporation emerges as a promising modality in tissue regeneration, offering the potential to enhance the precise delivery of therapeutic agents to cells and tissues. While additional investigations are required to comprehensively evaluate the safety and effectiveness of this method, its prospects are encouraging for advancing novel treatments across various conditions (26) . In order to promote the absorption of exogenous gene/drug molecules into cells, interactions between cavitation bubbles and live cells would result in "sonoporation," which is characterized by the brief rupture of cell membrane integrity (27) . Owing to the rarity of gaseous cavitation nuclei in the human body, sonoporation seldom occurs in healthy biological tissues or blood vessels. Synthetic microbubbles are frequently injected intravenously into the circulatory system to help initiate sonoporation. These microbubbles serve a dual purpose, of contrast agents in ultrasound image reporting and as agents in sonoporation procedures. These microbubbles typically have a gas-filled core (such as per fluorocarbon) enclosed in a thin stabilizing layer (such as lipid, albumin, or polymer); with an average size between 1 and 8 m and they may pass through the pulmonary capillary bed (28). It is well known that the presence of synthetic microbubbles significantly increases acoustic energy absorption and reduces the cavitation threshold, enhancing the severity of cavitation-induced bioeffects. Consequently, a wide range of therapeutic applications have been developed, leveraging the findings from in vitro and in vivo studies on gene and drug delivery. These applications span areas such as cancer therapy, opening of the blood-brain barrier, neurostimulation, treatment of cardiovascular diseases, and management of persistent bacterial infections (29).

Table 2: The utilization of sonoporation in the field of tissue regeneration.

Model of tissue regeneration	Animal experimental model	References
Bone Regeneration	Mouse	(30)
Peripheral Angiogenesis	Rabbit	(31)
Cardiovascular Angiogenesis	Mouse	(32)
Bone and Soft Tissue Integration	Pig	(33)
Islets of the Pancreas Regeneration	Rat	(4)

3.1. Utilizing Sonoporation for Pancreatic Islet Regeneration

Sonoporation involves the application of sound waves to induce transient openings in cellular membranes, enabling the targeted delivery of therapeutic genes or molecules. In the specific context of regenerating pancreatic islets, sonoporation offers a potential avenue for introducing genes that facilitate the proliferation and specialization of pancreatic stem cells, leading to the development of insulin-producing β-cells (34) . Pancreatic islets are clusters of cells in the pancreas that produce and secrete hormones such as insulin. Loss or dysfunction of these cells is the underlying cause of diabetes (35) . Therefore, the regenerating or replacement of these cells is a potential therapeutic strategy for diabetes. Using sonoporation, therapeutic genes can be delivered directly into pancreatic stem cells to promote their differentiation into β- cells. This technique has shown promise in preclinical studies, as it has been able to generate new β-cells in diabetic animal models (36).

In the pursuit of addressing diabetes mellitus, pancreatic islets have emerged as a therapeutic

target due to their composition of various cell types, including insulin-producing β (beta) cells. In 2010, groundbreaking research introduced the concept of ultrasound-targeted gene therapy as a potential means to regenerate pancreatic islets without the need for viral vectors, offering the possibility of curing diabetes (37) . Chen et al. conducted experiments utilizing a rat model of diabetes induced by streptozotocin (STZ), where they employed lipid stabilized microbubbles coupled with a rat insulin promoter (RIP3.1) that is modified specifically target β-cells. Multiple genes were tested, including Ngn3, PAX4, Nkx6.1, Nkx2.2, and Mafa. However, while some genes led to an increase in alpha-cell population, the β-cells and serum blood glucose levels did not exhibit significant changes 30 days after the sonoporation procedure (38). On the other hand, injection, named RIP3.1-NeuroD1 is found to prompt islet regeneration and restoration from surviving β-cells, results in the normalization of glucose, insulin, and C-peptide levels after 30 days of therapy. In a long-term trial, four out of six rats experienced β-cell apoptosis and the recurrence of diabetes after 90 days (39) . Notably, rats pretreated with the JNK inhibitor SP600125 exhibited an extended duration of islet regeneration and maintained normal blood glucose levels, suggesting the need for further investigation into immunosuppressive regimens for islet protection (33) . To achieve long-term transgenic expression of the Nkx2.2 gene in the pancreas of adult diabetic rat models, a piggyBac transposon gene delivery method was employed to deliver islet transcription factor genes (40) .The Nkx2.2 gene showed a strong capability to enhance the proliferation and differentiation of adult pancreatic precursors. Using high quality images, researchers were able to capture the progression of a single differentiated pancreatic precursor cell from islet cell like clusters to mature islets cell with characteristic cell features (42).

Remarkably, the effects of streptozotocin (STZ) on pancreatic islets in the body could be reversed over a period of three months. Recent studies have demonstrated that delivering plasmid named cyclin D2/CDK4/GLP-1 to the pancreas of STZ-treated rats can induce the re-entry of G0-phase of cell of islet of pancreas into the G1/ stage of the cell cycle, promoting cell regeneration (41) . A single sonoporation therapy without any sign of toxicity or activation of the oncogenes led to β-cell renewal and the diabetes is reversed for a duration of six months. The delivery of cyclin D2, CDK4, and GLP-1 stimulated the proliferation of adult pancreatic progenitor cells residing in the islets. Similarly, the delivery of the ANGPTL8 gene to the pancreas, liver, and skeletal muscle of healthy adult rats has been explored. ANGPTL8 was detected in the bloodstream one month after therapy. In a rat model of STZ-induced diabetes, sonoporation with ANGPTL8 resulted in significant improvement but did not completely normalize the condition (43).

Notably, targeting ANGPTL8 specifically to the pancreas yielded the most pronounced benefits, including increased numbers of mature and adult cells, leading to better glucose tolerance, and fasting insulin levels in blood without significant exacerbation of hypertriacylglycerolemia. However, the efficacy of using ANGPTL8 to treat diabetes in animal models has not been conclusively established. Experimental procedures involved injecting STZ to induce diabetes in three-month-old male rats, followed by the administration of pRIP3.1-ANGPTL8 or UTMD-pXL-BSII-CI-ANGPTL8/hyPB treatment, depending on the target organ. **(Figure 3)** demonstrates that both groups receiving ANGPTL8 therapy exhibited considerably lower fasting blood glucose levels compared to the STZ + UTMD-DsRed control group. However, these therapy groups still exhibited diabetes compared to healthy controls **(Fig. 3e).** Researchers speculate that the failure to fully correct the condition in the treated animals may be attributed to an insufficient number of cells capable of undergoing replication in significant quantities (44).

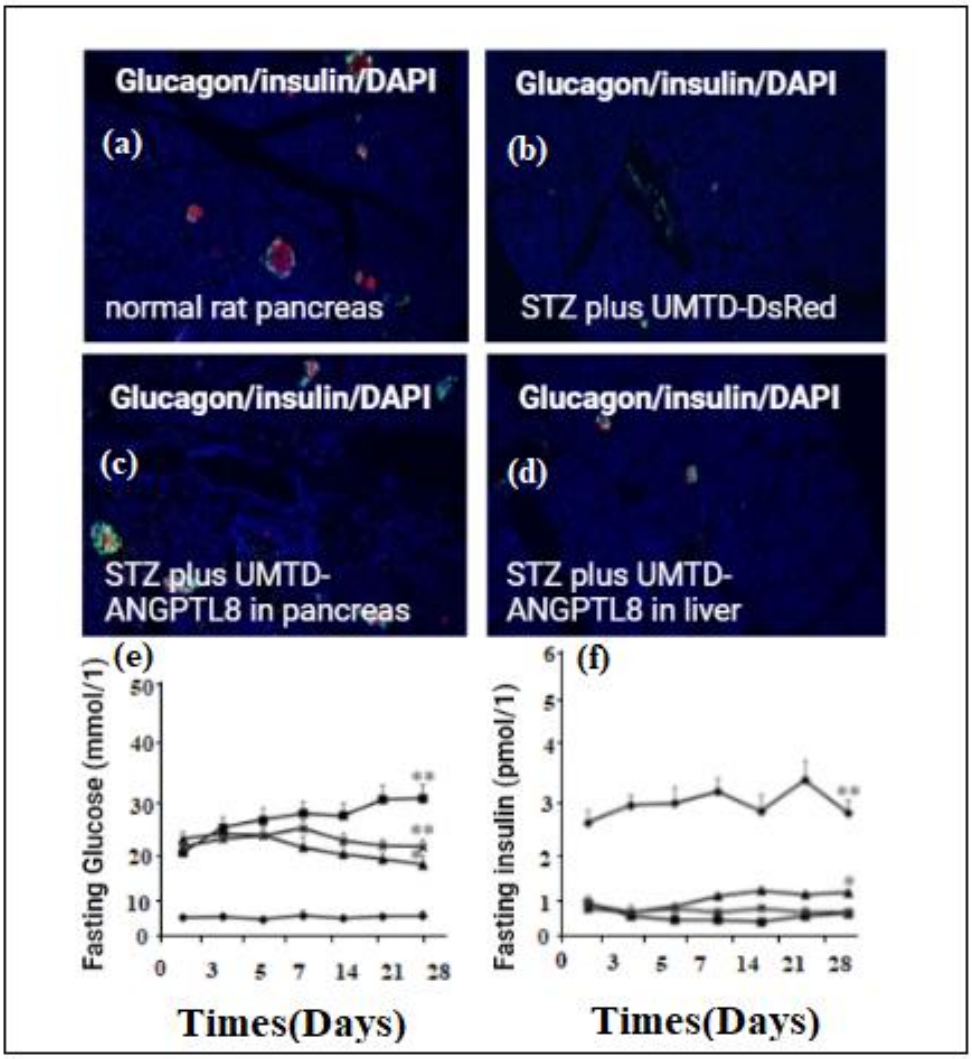

Figure 3 illustrates the evidence of islet regeneration in streptozotocin (STZ)-induced diabetic rats following ultrasound-targeted microbubble destruction (UTMD) with ANGPTL8. The images show the pancreas of a normal control rat (a), the pancreas with STZ-induced diabetes treated with UTMD-pRIP3.1-DsRed (b), the pancreas with STZ-induced diabetes treated with UTMD-pRIP3.1-ANGPTL8 (c), and the liver with STZ-induced diabetes treated with UTMD-pXL-BSII-CI-ANGPTL8/pCI-hyPB (d). Scale bars indicating 500 μm are provided. Fasting blood insulin levels (e) and blood glucose levels (f) are shown for different treatment groups: STZ plus UTMD-pRIP3.1-ANGPTL8 (black triangles), STZ plus UTMD-pRIP3.1-DsRed (black squares), STZ plus UTMD-pXL-BSII-CI-ANGPTL8/pCI-hyPB in the pancreas (black crosses), and normal controls (black diamonds). The data, presented as mean ± SEM, include six rats per group. Statistical analysis (*p < 0.05 and **p < 0.001) was performed to compare the different groups with the control groups.

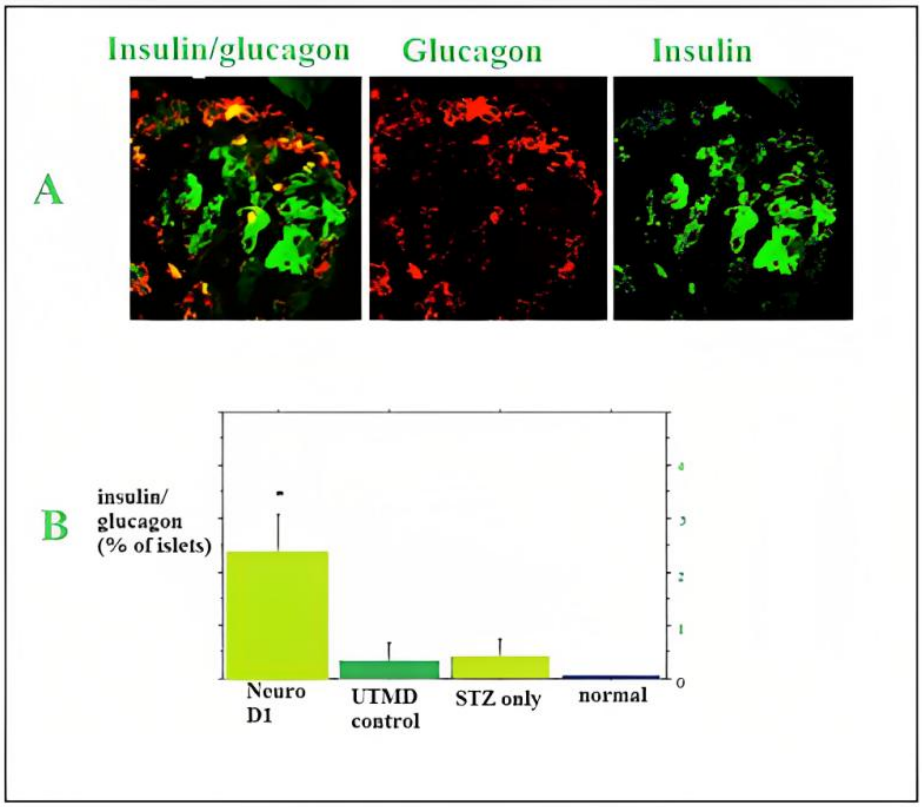

Figure 4 presents an example islet from a rat that has been treated with NeuroD1, as shown in the upper panels. The left panel displays the confocal imaging of the islet, with anti-insulin staining (green) and anti-glucagon staining (red) depicted. The colocalization of insulin and glucagon in specific cells (appeared yellow) in the confocal image indicates endocrine proliferation. The graph (B) illustrates the frequency of insulin and glucagon colocalization in rats treated with NeuroD1 compared to untreated rats, showing a significant increase in colocalization in the NeuroD1-treated group (p < 0.0001).

An example islet from a rat treated with NeuroD1 seen in panels (Figure.4). Overall, sonoporation holds potential as a non-invasive and targeted approach for regenerating pancreatic islets and treating diabetes. However, extensive research is required to determine the safety and efficacy of this technique in human patients.

3.2 Sonoporation as a Therapeutic Approach for Peripheral Ischemia

Peripheral ischemia is characterized by reduced blood flow to the peripheral tissues, typically in the limbs, which can result in pain, tissue damage, and impaired function. Sonoporation is a method that uses ultrasound waves to temporarily increase the permeability of cell membranes, allowing therapeutic agents to enter cells more easily. Approximately 200 million individuals are thought to have PAD worldwide, with the elderly making up a larger number of those affected. Owing to rising rates of obesity, type 2 diabetes, and a sedentary lifestyle, the prevalence of PAD increases with age (up to 20% in people over 65 years65years of age) (45) . Peripheral artery disease can't be cured through self-regeneration because of the same biological restrictions that prevent myocardial infarction from being cured. Sonoporation has been investigated as a potential treatment for peripheral ischemia by delivering therapeutic agents directly to the affected tissues.

For instance, scientists have utilized sonoporation to deliver growth factors like vascular endothelial growth factor (VEGF) to ischemic muscle tissue, promoting the formation of new blood vessels and enhancing blood flow (46) . The process of sonoporation involves the emission of high-frequency sound waves by a transducer, which generates microbubbles within the tissue. These microbubbles transiently open the cell membrane, facilitating the entry of therapeutic agents into the cells (47). Animals subjected to ultrasonic treatment exhibited significantly higher capillary density and angiographic scores compared to those receiving only HGF plasmid injections, five weeks after the procedure. Moreover, there were significant improvements in blood flow and blood pressure ratios (1) . The findings suggest that sonoporation-based gene therapy could be a safe and effective approach for treating peripheral artery disease. In

another study conducted on a rat model with severe chronic hindlimb ischemia, intravenously administered VEGF-165 plasmid in combination with ultrasonic treatment showed significant enhancements in microvascular blood flow and vessel density (48) . The increase in tissue blood flow was attributed to the elevation of capillary blood volume outside capillaries (arteriogenesis), peaking at 14 days post-therapy and partially regressing at 6 weeks (49) . The transfection primarily occurred in the vascular endothelium of arterioles. Subsequently, the efficacy of treatment was evaluated using intravenous (IV) and intramuscular (IM) injection sites in a similar experimental design. They discovered that microvascular blood volume and flow increased significantly following both IM and IV administration, however even eight weeks after therapy, the microvascular blood flow was higher in the IV-treated mice. It's interesting to note that animals treated with IM had considerably higher levels of VEGF165/GFP mRNA expression. The increased level of angiogenesis, in this case, may be explained by the guided vascular transfection across a larger distribution that occurred with intravenous infusion (50).

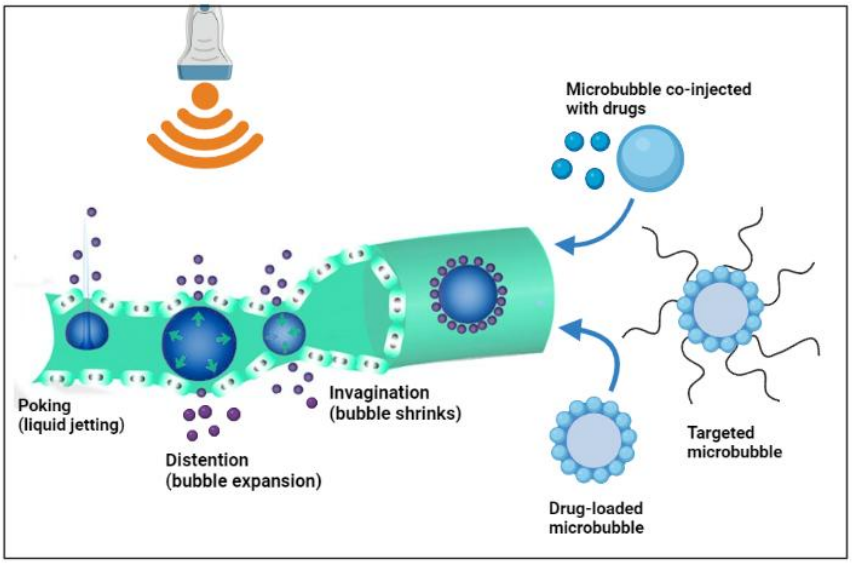

Figure5.Sonoporation has demonstrated a great efficacy in enhancing the uptake of drugs by inducing microbubble-mediated increase in the permeability of microvasculature. Created with BioRender.com

In a recent study, the combined effect of vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1) was compared to individual VEGF treatment for therapeutic angiogenesis in unilateral hindlimb ischemia in rat model. The researchers discovered that VEGF enhanced blood flow and vascular density when given in combination with cationic microbubbles (51). However, the the flow stayed modest, and the coverage of supporting cells in the newly formed channels was found suboptimal. To address this, the distribution of VEGF and Ang-1 was temporarily separated. VEGF was delivered at 2 weeks after ligation, followed by Ang-1 administration at 4 weeks post-ligation (52) .This method resulted in improvement in pericyte coverage at 8 weeks, results in increased blood flow and vascular density, and sustained flow reserve. In another study by Cao et al. in 2015, sonoporation was employed using miR-126-3p and cationic microbubbles in rats with chronic left femoral artery ligation (53). The authors hypothesized that manipulating VEGF and Ang-1 signaling pathways and inhibiting phosphatidylinositol-3-kinase regulatory subunit 2 (PIK3R2) and sprouty-related protein-1 (SPRED1) contributed to the observed biological effects (54) .The findings of the study also demonstrated that cationic microbubbles improved the stability and prolonged circulation in vivo. Furthermore, when the microbubbles were conjugated with miRNA prior to injection, they exhibited longer circulation time compared to unbound miRNA. Several studies have shown that sonoporation can improve blood flow and promote tissue regeneration in animal models of peripheral ischemia. For example, one study showed that sonoporation-mediated delivery of VEGF to ischemic muscle tissue in rabbits resulted in increased blood flow and improved muscle function (55). Overall, sonoporation has shown promise as a potential treatment for peripheral ischemia by

delivering therapeutic agents directly to the affected tissue. However, new research should be conducted to determine the safety and effectiveness of this technique in human patients.

Table 3 this demonstrates the application of sonoporation in the treatment of peripheral tissue ischemia.

Experimental Design	*Animal Species*	*Ultrasound Parameters*	*Frequency (MHz)*	*Findings*	*References*
				The infusion of VEGF-165 plasmid resulted in a significant improvement in microvascular blood flow and an increase in vessel density. The transfection mainly occurred in the vascular endothelium of arterioles. This finding suggests that VEGF-165 plasmid administration has a positive impact on vascular function and angiogenesis.	(56)
Hamstring ischemia	*Rat*	*S3 transducer and Philips Sonos 5500*	*1.3*	*Significant increases in microvascular blood volume and blood flow were observed with both intramuscular (IM) and intravenous (IV) administration of the VEGF-165 plasmid. However, the animals treated with IV administration showed higher microvascular blood flow compared to the IM-treated group. These findings suggest that IV administration may have a more pronounced effect on enhancing microvascular blood flow in the context of VEGF-165 plasmid delivery.* *Delivering VEGF and Ang-1 plasmids separately for a short period of time enhanced blood flow, vascular density, and maintained flow reserve.* *Microvascular perfusion*	(13)

				significantly improved after miR-126-3p therapy, and subsequent treatments produced even larger angiogenic responses.	

3.3 Exploring Sonoporation for the Treatment of Myocardial Ischemia

Myocardial ischemia happens when there is inadequate blood flow towards the heart, depriving the heart muscle of oxygen (57). Usually, a partial, semi partial or total blockage of your heart's arteries is what causes the lower blood flow (coronary arteries). The heart's capacity to pump the blood is decreased by myocardial ischemia, also known as cardiac ischemia. Heart attacks can occur as a result of an abrupt, severe blockage of a heart artery. Serious irregular heartbeats may also result from myocardial ischemia (58). The limited blood flow resulting in scar tissue formation and subsequent necrosis significantly hampers the process of cardiac wound healing in mammals (59).
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In a study conducted in 2012, ultrasound and microbubbles were utilized to deliver thymosin beta 4 (TB4) genes, carried by a piggyBac transposon plasmid, to the hearts of healthy rats. The objective was to address the limited self-regeneration capacity of cardiac tissue (60). The findings showed that local WT1-positive adult cardiac precursor cells proliferated and differentiated into three different cardiac cell lineages—cardiac muscle cells, coronary artery smooth muscle cells, and vascular endothelial cells—as a result of TB4 stimulation (59) . Additionally, it promoted arteriogenesis and angiogenesis (61) . The first research to show that sonoporation can be used for heart regeneration was published in 2009; however, this study supported its usage.

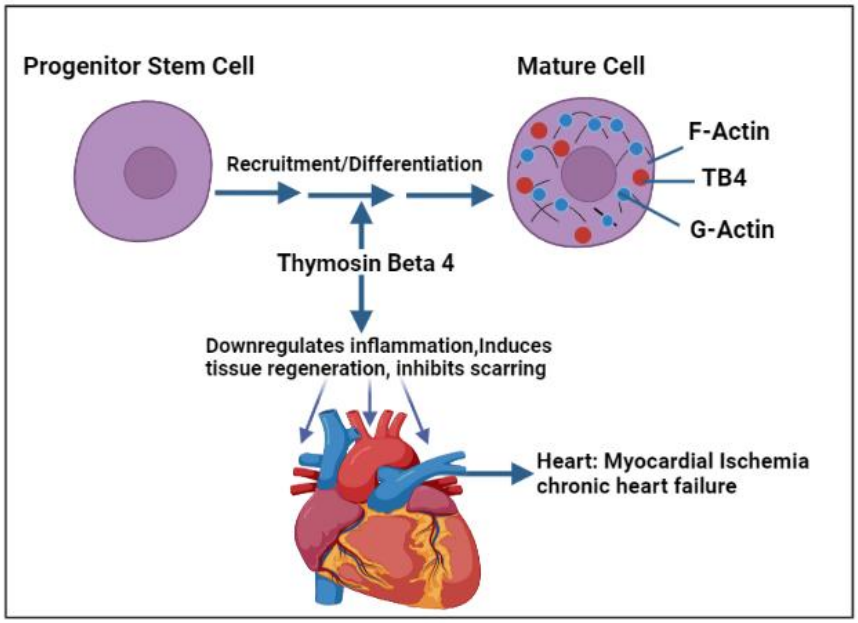

Figure6. This Figure shows the importance of thymosin beta 4 (TB4) in aiding heart neovascularization, increasing cell proliferation and differentiation, and preserving myocardial function. Created with BioRender.com

In order to simulate a myocardial infarction, in this 2009 study, mice were given intravenous injections of lipid microbubbles and plasmid DNA seven days after coronary artery ligation (MI). The implanted plasmid encoded either stem cell factor (SCF) or vascular endothelial growth factor (VEGF) (56). In comparison to the control group that received empty plasmids, the study revealed that the injection of either plasmid resulted in improved capillary and arteriolar density, myocardial perfusion, and cardiac function. Subsequently, the same researchers conducted a follow-up investigation to evaluate the effects of different therapies in a rat model of myocardial infarction (MI) (62) . The findings showed that all sonoporation-treated animals exhibited increased vascular density and reduced infarct size, but multiple injections of stem cell factor (SCF), stromal cell derived factor-1 (SDF-1) resulted in the greatest enhancement of vascular densities compared to the control group. Moreover, as the number of treatments increased, there was a gradual improvement in myocardial perfusion and ventricular function (63).

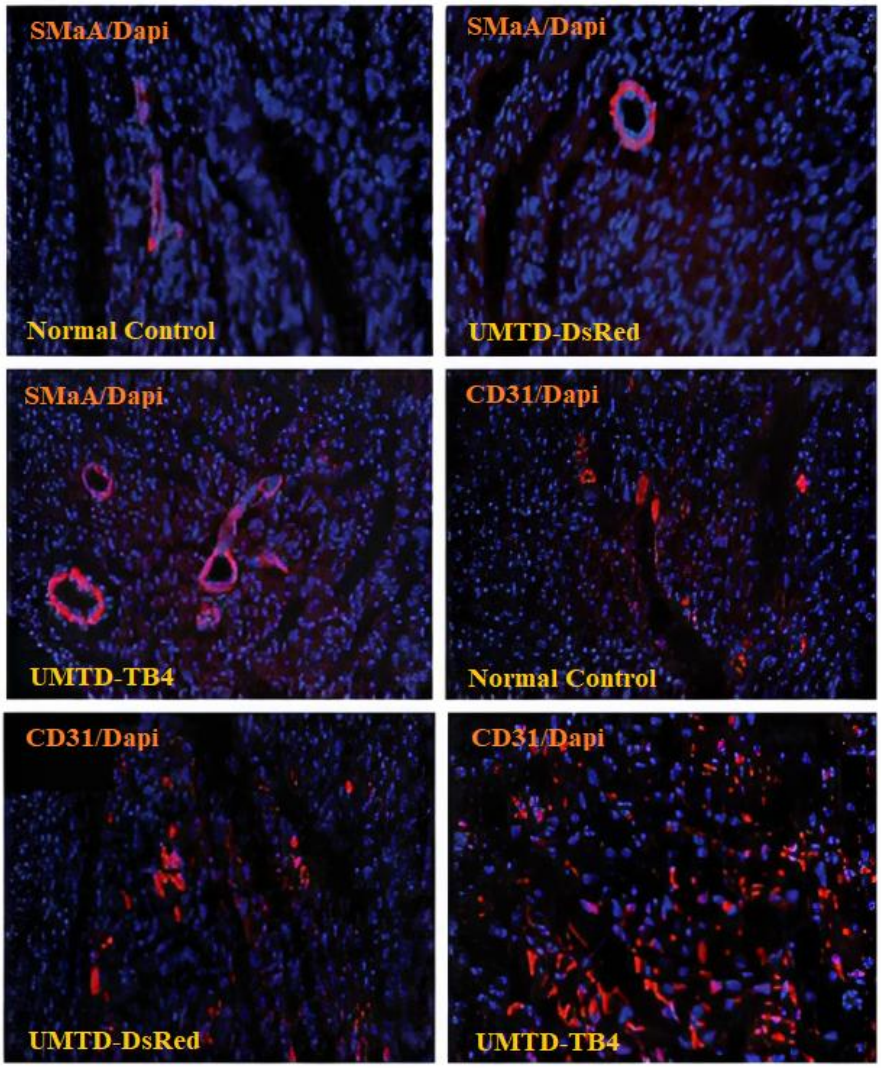

Figure 7 illustrates the angiogenesis observed after ultrasound-targeted microbubble destruction (UTMD)-mediated thymosin beta 4 (TB4) treatment, as depicted in representative microscopic images. The images display staining of smooth muscle alpha-actin (SMaA) in red and nuclei in blue. The upper panels present the staining patterns for the normal control, DsRed control, and UTMD-TB4 treatment groups from left to right, respectively. The scale bar provided in the images measures 150 μm. In the UTMD-TB4 therapy group, SMaA levels increased, which is associated with an increase in coronary arteriogenesis response. ((d)–(f)) Aside from the antibody being against CD31, the middle panels are similar.

A more recent study aimed to improve the delivery of antagomir to the heart using ultrasound and cationic microbubbles (69) . Previous studies used plasmid injections, but antagomir, a microRNA inhibitor, was chosen in this study due to its weak myocardial selectivity and potential side effects when used in large doses during cardiac therapy (65) . The researchers found that ultrasound and cationic microbubbles greatly enhanced the local delivery of antagomir to the non-ischemic heart. They observed minimal side effects, such as neutrophil infiltration, without an increase in apoptosis. The study revealed that antagomir reached cardiomyocytes within 30 minutes of therapy and remained present for at least 48 hours (66) . However, the ultrasound data did not show additional regeneration benefits in the infarcted zone after ischemia-reperfusion damage. Interestingly, the frequency and mode of ultrasound had an impact on the amount and distribution of antagomir delivery. Higher frequencies resulted in more constrained distribution to the anterior wall, while lower frequencies allowed delivery to other areas of the heart (64). The study demonstrated that sonoporation was safe and did not directly harm cardiomyocytes, indicating its potential for

preventing cardiac tissue destruction. Another study investigated the use of sonoporation for delivering therapeutic genes to a myocardial infarction (MI) rat model using microbubbles conjugated to adenoviruses encoding for the SERCA2a and Cx43 genes (71) .The mice receiving both genes showed the highest cardiac contractile performance and electrical stability compared to the control group (70). Interestingly, the therapeutic effectiveness was significantly enhanced when bone marrow mesenchymal stem cells (MSCs) were injected before sonoporation, suggesting that the MSCs assisted in maintaining a population of cardiomyocytes intended for transfection (67) .The study highlights the potential advantages of combining sonoporation with other gene delivery technologies and emphasizes the importance of targeting the appropriate responder cells for regeneration (68) (72) . Although Table 4 lists studies using sonoporation for heart tissue regeneration, further research is still needed in this field.

Table 4 illustrates the utilization of sonoporation for the purpose of regenerating heart tissue.

Experimental Model	*Animal Species*	*Ultrasound Application*	*Frequency of ultrasound in (MHz)*	*Findings*	*Sources*
Damage from ischemia/reperf-usion (I/R)	*Rat*	*Ultrasound System: The Siemens Acuson Sequoia C256 ultrasound system was used. Ultrasound Transducer: The 15L8 transducer was utilized in the study.*	*8*	*The delivery of the AKT gene using cationic microbubbles resulted in the most significant enhancement in ventricular function and myocardial perfusion, accompanied by a decrease in infarct size and apoptosis.*	(56)
		Transducer: M3S; GE Healthcare Vivid 7	*1.6*	*The frequency and mode of the ultrasound are crucial for antagomir distribution to the myocardium, which mainly affects the anterior wall of the heart.*	(56)

3.4 Utilizing Sonoporation for Skeletal Tissue Regeneration

Skeletal tissue regeneration is a complex process that relies on the coordinated activity of various cellular and molecular factors (73) .Traditional treatment approaches for skeletal tissue injuries and disorders often have limited success in fully restoring tissue structure and function. However, emerging techniques such as sonoporation offer promising prospects for enhancing tissue regeneration. Sonoporation utilizes ultrasound and microbubbles to deliver therapeutic agents, genes, and growth factors to target tissues,

enabling precise and efficient delivery (74). This article, will explore the application of sonoporation in skeletal tissue regeneration and discuss relevant experimental studies that highlight its potential. One notable study investigated the use of sonoporation to enhance bone regeneration in a rat model. In this experiment, cationic microbubbles were loaded with bone morphogenetic protein-2 (BMP-2) and administered locally to a bone defect site in the rat femur. Ultrasound was applied to the target area to induce microbubble cavitation and facilitate the delivery of BMP-2. The results showed that the sonoporation group exhibited accelerated bone healing, increased bone mineral density, and enhanced bone formation compared to the control group (76). Cartilage repair is another crucial aspect of skeletal tissue regeneration (75). A study focused on using sonoporation to deliver therapeutic genes for cartilage repair in a rabbit model. The researchers employed ultrasound and microbubbles loaded with insulin-like growth factor-1 (IGF-1) plasmid to the damaged cartilage site. Sonoporation-mediated delivery of IGF-1 resulted in improved cartilage regeneration, increased chondrogenic gene expression, and enhanced extracellular matrix synthesis compared to control groups (83).Tendon injuries often pose significant challenges for effective healing. In a study investigating the potential of sonoporation in tendon healing, a rat model of Achilles tendon injury was used. Ultrasound and cationic microbubbles loaded with connective tissue growth factor (CTGF) plasmid were employed to deliver CTGF to the injured tendon site. Sonoporation-mediated CTGF delivery led to accelerated tendon healing, improved collagen organization, and increased mechanical strength of the repaired tendon compared to the control group (77). Muscle regeneration is vital for restoring functional movement in cases of muscle injuries or degenerative conditions (78). A study focused on investigating the application of sonoporation for muscle regeneration in a mouse model. In this experiment, ultrasound and microbubbles loaded with plasmid containing hepatocyte growth factor (HGF) were locally injected into the damaged muscle tissue. Sonoporation-mediated HGF delivery resulted in enhanced muscle regeneration, increased muscle fiber size, and improved muscle function compared to control groups (79). The experimental studies discussed above provide evidence of the potential of sonoporation for enhancing skeletal tissue regeneration (82). These studies highlight the effectiveness of sonoporation in delivering therapeutic agents, genes, and growth factors to the target tissues, leading to accelerated tissue healing, improved tissue structure, and enhanced functional outcomes (13).Sonoporation offers several advantages, including precise spatial and temporal control of therapeutic agent delivery, non-invasive nature, and minimal side effects (80). However, it is important to note that further research is needed to fully understand the mechanisms underlying sonoporation-mediated tissue regeneration and optimize its parameters for different tissue types and injury conditions (81). Additionally, clinical studies are required to validate the efficacy and safety of sonoporation in human patients (84). Nonetheless, the potential of sonoporation in the regeneration of skeletal tissue holds great promise for the development of novel therapeutic strategies in the field of regenerative medicine.

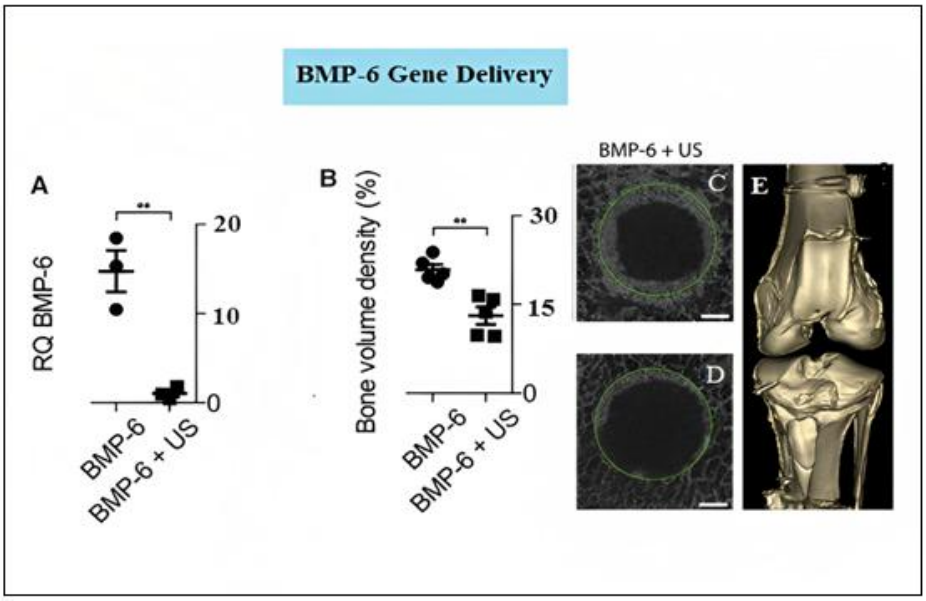

Figure 8 illustrates the delivery of the BMP-6 gene to mini-pig anterior cruciate ligament (ACL) repair sites using ultrasound. Panel A shows the expression of the BMP-6 gene, measured by relative quantification (RQ), five days after treatment in the bone tunnels at the ACL repair sites. In panel B, a quantitative analysis of bone development in the reconstruction areas is presented at eight weeks post-surgery. Representative micro-computed tomography (mCT) slices of the bone tunnels, with and without ultrasonic therapy, are displayed in panels C and D, respectively. The green circles indicate the original widths of the bone tunnels created during surgery. The scale bars represent one millimeter. Panel E demonstrates a fluoroscopic 3D reconstruction of a typical knee joint treated with ultrasound and BMP-6.

Exploring the potential synergistic effects of combining sonoporation with other techniques to enhance gene transfer efficacy represents an exciting future direction in the field of sonodelivery. Table 5 gives brief summary of previoue studies tha utilized sonoporation for skeletal tissue regeneration. Despite the promising outcomes observed thus far, there is still a desire for improved transfection efficiency. Research suggests that modification in the ultrasound parameters can enhance gene expression in the defected bone model for a duration of consisting of almost 21 days.

Table 5 provides a compilation of studies demonstrating the utilization of sonoporation for the purpose of regenerating skeletal tissue.

Experimental Model	*Animal Species*	*Ultrasound Application*	*Frequency of ultrasound in (MHz)*	*Findings*	*Sources*
		The Rich-Mar Sonitron model from the year 2000.		*The combination of sonoporation, intramuscular injection of rhBMP-9 plasmid, and lipid-stabilized microbubbles resulted in the induction of ectopic bone formation.*	(85)

Ectopic Mouse	*Mouse*	*The Rich-Mar Sonitron model from the year 2000.*	*1*	*Significant enhancement of osteoinduction was observed when comparing two treatment sessions to repeated sonoporation using the BMP-2 plasmid.*	
		SP100 Sonidel		*The utilization of 4 W/cm2 sonoporation in conjunction with a constitutive BMP2/7 co-expression plasmid resulted in a significant increase in ectopic bone production, albeit with varying morphology and irregular shape.*	(86)
		SP100 Sonidel		*In contrast to conventional sonoporation, the combination of a GAM (guided acoustic wave) technique and BMP2/7 co-expression plasmid demonstrated remarkable enhancement in ectopic bone growth.*	
Femur Error	*Rat*	*SP100 Sonidel*	*1*	*While the observed outcome did not reach statistical significance, the utilization of a BMP2/7 co-expression plasmid resulted in fracture union in 33% of the rats, compared to 0% in the control group.*	(87)

Nevertheless, due to the high ultrasonic wave reflection caused by Implants made of bone and metal., such as those used to treat fractures, sonoporation is particularly difficult in these situations.

3.5 Exploring Additional Applications of Sonoporation in Tissue Regeneration

Sonoporation has shown promising potential in various aspects of tissue regeneration beyond the examples discussed earlier. This section explores additional uses of sonoporation in tissue regeneration, highlighting its versatility and broad applications. One area of interest is the regeneration of skin tissue (88) .Sonoporation has been investigated as a method to enhance the delivery of therapeutic agents for wound healing and skin regeneration. Studies have demonstrated that sonoporation can improve the transdermal delivery of growth factors, cytokines, and other therapeutic molecules, promoting accelerated wound healing and tissue regeneration. By creating transient pores in the skin, sonoporation enables more efficient uptake of these agents, enhancing their effectiveness in promoting tissue repair. In the field of bone tissue regeneration, sonoporation has been explored as a means to enhance the delivery of osteogenic factors and genes to stimulate bone

growth (89) . Through the use of ultrasound and microbubbles, therapeutic agents can be efficiently delivered to bone defects or fracture sites. Sonoporation has shown potential for promoting osteogenesis and bone regeneration in preclinical studies, offering a non-invasive and targeted approach for improving bone healing. Furthermore, sonoporation has been investigated for its potential in promoting the regeneration of cartilage tissue (90). Cartilage injuries and degenerative conditions pose significant challenges due to the limited regenerative capacity of cartilage. Sonoporation offers a promising strategy for delivering growth factors, stem cells, or genetic materials to promote cartilage repair. By enhancing the penetration and uptake of therapeutic agents, sonoporation may facilitate the regeneration of functional cartilage tissue and provide potential solutions for cartilage-related disorders. In addition to traditional tissue types, sonoporation has also been explored for the regeneration of neural tissue. Neurological disorders and injuries often result in limited recovery due to the inability of neural tissue to regenerate effectively (91) .Sonoporation-based approaches aim to overcome this challenge by facilitating the delivery of neurotrophic factors, stem cells, or gene therapies to promote neural regeneration. Preclinical studies have demonstrated the potential of sonoporation in enhancing the therapeutic effects for neurological conditions, including spinal cord injuries and neurodegenerative diseases (94) .Moreover, sonoporation has been investigated in the field of vascular tissue engineering. The creation of functional blood vessels is crucial for tissue regeneration and transplantation. Sonoporation has been utilized to enhance the delivery of angiogenic factors and stem cells to promote vascularization in tissue-engineered constructs (95).By facilitating the penetration and distribution of these therapeutic agents, sonoporation can enhance the formation of functional blood vessels, leading to improved tissue integration and survival (92). Overall, sonoporation holds great promise for tissue regeneration in various fields, including skin, bone, cartilage, neural tissue, and vascular tissue engineering. Its ability to enhance the delivery of therapeutic agents, genes, and stem cells makes it a versatile tool for promoting tissue repair and regeneration (93) . Continued research and development in this field are expected to further uncover the potential applications of sonoporation, ultimately advancing the field of tissue regeneration and improving clinical outcomes (96).

4. Guidelines for Clinical Translation

Ultrasound-assisted gene therapy has emerged as a promising approach for tissue regeneration, offering a non-invasive and targeted method to deliver therapeutic genes to the desired tissues. As this innovative technique moves towards clinical translation, it is crucial to establish guidelines to ensure its safe and effective implementation (97) . In this section, we present guidelines for the clinical translation of ultrasound-assisted gene therapy, considering various aspects such as gene selection, vector design, and optimization of ultrasound parameters, microbubble enhancement, safety considerations, and clinical trial design. One of the key considerations in clinical translation is the selection of therapeutic genes and vectors. Careful evaluation of the genes' regenerative potential and the vectors' transfection efficiency and immunogenicity is essential (98) . The chosen genes should possess the ability to promote tissue regeneration, while the vectors should offer efficient transfection and controlled release of therapeutic genes for optimal therapeutic effects. Optimization of ultrasound parameters is critical for successful clinical translation. Factors such as ultrasound frequency, intensity, duration, and mode should be carefully adjusted to achieve efficient transfection without causing tissue damage (99) . A thorough understanding of the interaction between ultrasound and target tissues is necessary to tailor the ultrasound parameters to specific tissue types and regenerative goals. Microbubble enhancement has shown great potential in improving gene delivery efficiency (100) . Optimizing microbubble characteristics, such as size, stability, and surface modification, can enhance gene transfer efficiency and tissue regeneration outcomes. The development of standardized and reliable microbubble formulations will be crucial for clinical translation. Safety considerations are paramount in the clinical translation of ultrasound-assisted gene therapy (101) . Comprehensive preclinical studies are necessary to assess potential adverse effects, including immune responses, off-target effects, and tissue damage. Long-term safety and potential toxicity should be carefully evaluated to ensure patient well-being during clinical trials. Monitoring and mitigation strategies should be in place to minimize risks and ensure patient safety. The design of well-controlled and well-designed clinical trials is essential for evaluating the efficacy and safety of

ultrasound-assisted gene therapy in tissue regeneration. Proper patient selection, standardized treatment protocols, and appropriate outcome measures should be established. Long-term follow-up assessments are necessary to monitor the durability of therapeutic effects and potential late complications (102).

Collaboration between scientists, clinicians, and regulatory authorities is crucial for the successful clinical translation of ultrasound-assisted gene therapy (103) .It is important to establish clear guidelines and protocols to facilitate the regulatory approval process and ensure compliance with ethical standards. Continuous research and knowledge exchange will further advance the field and improve the clinical implementation of this promising approach. In conclusion, ultrasound-assisted gene therapy holds significant promise for tissue regeneration. Establishing guidelines for its clinical translation is essential to ensure its safe and effective application (104). Considerations such as gene selection, vector design, and optimization of ultrasound parameters, microbubble enhancement, safety considerations, and well-designed clinical trials play crucial roles in the successful clinical translation of this innovative approach. With continued research and collaboration, ultrasound-assisted gene therapy has the potential to revolutionize tissue regeneration and improve patient outcomes.

5 Conclusion and Future Outlook

In conclusion, ultrasound-assisted gene therapy has emerged as a promising approach for tissue regeneration. This non-invasive and targeted method of delivering therapeutic genes to specific tissues holds great potential for treating various diseases and injuries. The studies reviewed in this article have demonstrated the effectiveness of ultrasound-assisted gene therapy in promoting tissue regeneration in different preclinical models. The use of ultrasound in combination with gene therapy offers several advantages, including enhanced gene delivery, improved transfection efficiency, and controlled release of therapeutic genes. Ultrasound can facilitate the uptake of therapeutic genes into target cells, overcoming barriers such as cell membranes and extracellular matrices. Additionally, the use of microbubbles as gene carriers further enhances gene transfer efficiency, allowing for more effective tissue regeneration. The reviewed studies have shown successful outcomes in various tissues, including bone, muscle, cartilage, and cardiac tissue. Ultrasound-assisted gene therapy has been demonstrated to promote angiogenesis, stimulate cell proliferation and differentiation, enhance tissue repair, and improve functional outcomes. These findings highlight the potential of this approach to revolutionize clinical treatments for tissue regeneration. Despite the promising results, there are still challenges and limitations that need to be addressed for the clinical translation of ultrasound-assisted gene therapy. Optimization of ultrasound parameters, such as frequency, intensity, and duration, is crucial to ensure safe and effective gene delivery while minimizing potential tissue damage. Standardization of microbubble formulations and characterization of their properties will further enhance gene transfer efficiency and clinical applicability. Safety considerations remain a priority in the development of ultrasound-assisted gene therapy. Comprehensive preclinical studies are necessary to assess potential adverse effects and ensure patient safety during clinical trials. Long-term follow-up assessments are needed to evaluate the durability of therapeutic effects and monitor for any late complications. Future research should focus on advancing our understanding of the underlying mechanisms involved in ultrasound-assisted gene therapy. Exploring the interactions between ultrasound, microbubbles, therapeutic genes, and target tissues will provide valuable insights for further optimization of this approach. Additionally, the development of more efficient gene delivery vectors and the identification of novel therapeutic genes will expand the potential applications of ultrasound-assisted gene therapy. Clinical translation of ultrasound-assisted gene therapy requires close collaboration between scientists, clinicians, and regulatory authorities. Establishing standardized protocols, guidelines, and ethical standards will facilitate the regulatory approval process and ensure the safe implementation of this innovative approach. Continued research, clinical trials, and knowledge exchange will further advance the field and pave the way for the widespread clinical application of ultrasound-assisted gene therapy for tissue regeneration. In conclusion, ultrasound-assisted gene therapy holds great promise for tissue regeneration. With ongoing advancements and collaborative efforts, this approach has the potential to revolutionize clinical treatments, improve patient outcomes, and offer new therapeutic options for various diseases and injuries.

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