

# Application of ultrasound microbubble contrast technology in ophthalmic targeted therapy: literature analysis

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## INTRODUCTION

With the completion of human genome project, people have placed the hope of radically curing many intractable diseases on gene medicines. However, current gene therapy still has many unsolved problems, such as lack of the safe, effective, organized, specific and targeted gene transshipment system, and lack of stable expression as well as the host reaction after transcription<sup>[1,2]</sup>. Besides, most current gene introduction ways are directly injecting the target gene into target tissue, having defects like being invasive and low amount of transduction which limit its clinical application.

In recent years, the new carrier-ultrasound microbubble contrast agent in the ultrasound field has achieved gratifying results in targeted therapy, which has made it draw more and more attention from the medical profession. Due to the blood-ocular barrier, the eyeball is relatively independent of the systemic circulation, the opportunity of viral vectors introduced into eyes affecting the organs outside the eye was reduced, thus the systemic side effects were minimized and the therapeutic effect was maximized at local ocular part. This advantage makes gene therapy receive widespread concern in the treatment of ophthalmic diseases. Under the irradiation by ultrasound with certain energy in the outside world, the ultrasound microbubble can mediate target gene or drug to achieve the purpose of targeted therapy<sup>[2-4]</sup>. To combine the new carrier-ultrasound microbubble contrast agent provides a new way for the gene therapy of ophthalmic diseases.

## MATERIALS AND METHODS

### Materials

**Ultrasound microbubble contrast agents** So far, the development of ultrasound microbubble contrast agent has

## Abstract

- **AIM:** To analyze the application of microbubble contrast technology in the treatment of ophthalmic diseases, mainly analyzing its advantages and existing problems.

- **METHODS:** A total of 30 representative literatures about the application of ultrasound contrast agent in gene targeted therapy at home and abroad were collected, and focusing on sorting out the literature reporting the treatment of ophthalmic diseases with microbubble contrast technology in recent years, then recalling its advantages and problems, finally making reasonable assessment on existing problems and proposing possible solutions to the problems.

- **RESULTS:** Due to its unique safety and efficacy, the treatment of ophthalmic diseases with microbubble contrast technology has increasingly drawn the attention of clinicians, but two relevant issues should be considered: first, the nature of contrast agent and the choice of corresponding ultrasound parameters; second, relative incidence of tissue bleeding, intravascular hemolysis, moderate or severe allergy as well as other side effects.

- **CONCLUSION:** Microbubble may become the carrier of targeted therapy, and as a kind of new non-invasive delivery system, the ultrasound contrast agent has broad application prospects, but its application in ophthalmic research is still in its initial stage and the safety of contrast-enhanced ultrasound still needs further study.

- **KEYWORDS:** microbubble contrast technology; targeted therapy; ophthalmic diseases

undergone three stages <sup>[1,2,5]</sup>. In the first stage, free gas without film-forming substances which is unstable for peripheral intravenous injection. It should insert into the aorta or the heart cavity through cardiac catheterization which is an invasive examination method. Besides, the microbubble has an extremely short duration in the blood circulation, and the agent's bubble formation is too big to pass the pulmonary circulation, thereby leading to the right ventricle developing without the left ventricle developing. As a result, its application is limited. In the second stage, albumin, lipids or polysaccharides are used as the membrane materials to wrap up air bubbles. Alunex and Levovist are the representatives of commercialized contrast agents, which have relatively stable membrane materials with smaller diameter ( $<8\mu\text{m}$ ), and significantly prolonged duration in the blood. Peripheral intravenous injection could not only enhance the right ventricle developing, but also could stably develop left ventricular cavity and peripheral vessels through the pulmonary capillary, achieving the development from invasive to non-invasive. In the third stage, the fluorocarbon gas or the fluorinated gas is used with larger molecule as well as low solubility and dispersion and bio-inert to replace air, oxygen, carbon dioxide, nitrogen and other gases, and new membrane materials with high resistance to pressure and stability like phospholipids, liposomes, non-ionic surface active agents as well as biodegradable macromolecule polymers were introduced, which will make the microbubble diameter of new generation ultrasound contrast agent shorter and become consistent with stable physicochemical properties and the stability and resistance to pressure will be further improved.

Most ultrasound microbubble contrast agents used currently are microbubbles containing inert gas and can suspend in the liquid. The composition of the shell can be human albumin, lipids, palmitic acid and polymers. The target gene combines with the ultrasound microbubble contrast agents through two ways: adhering to the shell surface by electrostatic effect or being wrapped up in the microbubble <sup>[6]</sup>.

**The mechanism of targeted therapy** After the ultrasound microbubble contrast agent was intravenously injected into the target tissue, it could effectively penetrate the endothelial barrier and directionally release the internally wrapped gene or drug due to the "cavitation effect" under ultrasonic energy effect, thus to make the local concentration greatly increase and achieve the purpose of targeted therapy. The fundamental principle <sup>[7-11]</sup> for the "cavitation effect" is that after the ultrasonic waves in the acoustic venue destructed the microbubble contrast agent, the generated cavitation or mechanical effect could improve the permeability of cell membrane, and make the capillaries with a diameter  $\leq 7$

microns break and the endothelial intercellular widen, thus to make the target gene or drug reach the tissue cells through broken capillaries and endothelial intercellular. At the meantime, the ultrasonic wave could break the microbubbles within the target tissue at a specific time and space, improving the targeting of treatment, increasing the local concentration of the gene or drug, enhancing its efficacy, simultaneously reducing the consumption of the gene or drug before reaching the lesion, and reducing the side effects on normal tissues. More favorably, the ultrasonic testing could synchronously provide in real time the pathophysiological information on anatomy structure, blood flow and blood perfusion function and metabolism as well as other tissues and organs. The ultrasonic tissue characterization could reflect certain features of the tissue. Tissue contrast echocardiography could quantify the local tissue blood flow, so the therapeutic effect of the gene or drug could be evaluated through observing the changes of the sonogram before and after the treatment of the disease.

The eyeball is an ideal organ for local gene therapy, as it needs small tissue operating space and relatively low drug concentration, thus it can maximally reduce the effective drugs diffusing to other organs beyond the eye. Meanwhile, the eyeball is in a state of relative immune privilege, so intraocular injection of exogenous antibodies could minimally cause potential immune response and inflammation <sup>[12]</sup>. However, the shortcomings of traditional gene vectors such as insecurity, poor targeting and complicated drug delivery present difficulties for the application of gene therapy in ophthalmology. Some scholars introduce ultrasound microbubble into the gene therapy for ophthalmic diseases because of its good targeting, high safety and high transfection efficiency and *etc.*, mainly focusing on the research of the corneal and retinal diseases currently.

**Methods** With the extensive researches on the ultrasound microbubble contrast agent in gene transfection and gene therapy, many scholars also have introduced ultrasound microbubble contrast agent into ophthalmology and conducted a preliminary exploration. At present, the researches on ophthalmic gene transfection were reported successively, so a further experimental study on the transfection of eye tissue cells could be made in order to find appropriate ultrasonic irradiation parameters, which will provide new means for the gene therapy on ophthalmic diseases.

In this article, 32 literatures about the role of ultrasound microbubble contrast agent in gene targeted therapy were collected from domestic and foreign reports from PubMed database and Wanfang database, including 10 domestic articles (accounting for 31.25%) and 22 foreign articles

(accounting for 68.75%), and a retrospective analysis of the above references was made. The goal of this retrospective analysis was the role and effect as well as its safety *etc* of ultrasound contrast agent in gene targeted therapy. Fourteen references (43.75%) reporting the researches on the ultrasound microbubble contrast agent in the ophthalmic gene targeted therapy were selected from the above references, and this paper mainly focused on analyzing the specificity of ultrasound contrast agent in the ophthalmic gene targeted therapy as well as the prediction of its role and effect. Meanwhile, a full range of analysis of the problems existing in relevant researches was made, thereby providing the theoretical basis for the ultrasound contrast agent in the ophthalmic gene targeted therapy.

Based on elaborating the role of ultrasound microbubble contrast agent in gene transshipment, an analysis was conducted from three aspects such as corneal diseases, retinal diseases and ocular tumors in this article. At the meantime, a targeted analysis made on each aspect involving the research findings of *in vivo* and *in vitro* experiments about the role of ultrasound microbubble contrast agent in gene targeted therapy, furthermore obtaining comprehensive and empirical experimental evidence.

**Corneal diseases treatment** Sonoda *et al* [13] used the ultrasound+microbubble contrast agent mediating green fluorescent protein (GFP) gene to transfect the rabbit corneal epithelial cells, and screened the best ultrasonic irradiation condition. It was found that the transfection efficiency of the ultrasound + microbubble contrast agent+plasmid group was significantly higher than the plasmid+ultrasound group; while there were almost no GFP expressions in the ultrasound microbubble+plasmid group and the naked plasmid group. In the *in vitro* experiments, the acoustic intensity of 0.5-1.0W/cm<sup>2</sup>, the microbubble concentration of 20%, the irradiation time of 60-120 s and the air proportion of 50% were selected as the best irradiation conditions for the ultrasound microbubble transfection. In the *in vivo* experiments the results showed that there werenot many GFP-positive cells in the naked plasmid group and the ultrasound microbubble + plasmid group, a little more in the plasmid + ultrasound group, while more in the ultrasound + microbubble contrast agent + plasmid group under the acoustic intensity of 1W/cm<sup>2</sup> and 2W/cm<sup>2</sup>. Besides, it was observed that all GFP-positive cells distributed around the injection area, mainly in the corneal cells. There was GFP expression on the second day after the transfection, and then the expression gradually increased. It reached the peak on the eighth day after the transfection and then gradually decreased, and there was only weak expression 30 days after the transfection. It was observed from the experiment that

different acoustic intensity (0.5-3W/cm<sup>2</sup>) had no damage to tissue cells.

Yamashita *et al* [14] used a new lipid microbubble+ultrasound mediating GFP gene to transfect *in vitro* cultured rabbit corneal epithelial cells (RC-1), and compared with traditional microbubble, the results showed that transfection efficiency of the gene in this new lipid microbubble group was significantly higher than other control groups, and no obvious cell damage was seen simultaneously. Hu *et al* [15] found that the ultrasound combined with liposome microbubble could significantly enhance the cavitation effect of *in vivo* rabbit corneal tissue, and the greater the ultrasonic energy was, the more severe the damage to the corneal tissue. At the meantime, it was also found that the ultrasonic acoustic intensity of 0.15W/cm<sup>2</sup> and the irradiation time of 30s or 60s might be appropriate for the corneal tissue.

**Gene therapy on retinal diseases** Many studies [16-18] showed that there existed blood-retina barrier damage at the time of the occurrence of retinal pathological changes, and this mainly reflected in: 1) Ultrastructural changes in barrier; 2) Changes in permeability. The pathological changes in the blood-retina barrier provided a structural basis of tissue pathology for macromolecules like the ultrasound microbubble entering into the pathological tissue through blood vessels.

Currently, the gene therapy on retinal diseases with ultrasound microbubble mainly focused on the study of choroidal neovascularization (CNV) [1,2,19,20], chronic ocular hypertension in the nerve cell damage and other aspects. Zhou *et al* [21] proved in the experiment that using the low-frequency ultrasound + microbubble contrast agent with certain energy could effectively improve the transfection efficiency of epithelium-derived factor plasmid in the rat retina and choroid and make it efficiently express as well as inhibiting ratchoroidal neovascularization to a certain degree. Li *et al* [22,23] cultured the retinal ganglion cells (RGC) *in vitro* and used ultrasound microbubble contrast agent mediating GFP gene to transfect RGCs, and the results showed that the transfection efficiency of the ultrasound+ microbubble contrast agent+plasmid group was significantly higher than the ultrasound+plasmid group (about 5 times). Fischer *et al* [24] successfully used ultrasound microbubble mediating pCAX-eGFP to transfect RGCs, photoreceptor cells and chick retinal neurons including bipolar cells, and the study confirmed that the ultrasound+microbubble contrast agent+plasmid group had a higher survival rate and higher transfection efficiency.

As to *in vivo* studies, Xu *et al* [25] used ultrasound microbubble contrast agent mediating enhanced EGFP plasmid to transfect Long-Evens rat retina and choroid, and

the experiment was divided into the ultrasound group, the microbubble+ultrasound group, the naked plasmid group, the ultrasound+plasmid group, the microbubble+plasmid group and the ultrasound+microbubble+plasmid group. The ultrasonic acoustic intensity was  $0.5\text{W}/\text{cm}^2$ , and the drugs were injected through tail vein, and the EGFP expressions were observed 2 weeks after transfection. The results showed that EGFP expressed on the rat retina in each group, mainly in the pigment epithelium layer, and the expressions in the ultrasound+microbubble+plasmid group were highest, which had statistical significance compared with other groups by statistic analysis. Under the ultrasonic effect with certain frequency and energy, the transfection efficiency of the target gene could effectively improved by ultrasound microbubble mediating, and rat pigment epithelium could be transfected.

Hirokawa *et al* [26] used the harmonic ultrasound contrast with low mechanical index (MI) to observe the uveal perfusion in New Zealand white rabbits. The results showed that the uveal blood signal intensity was significantly enhanced after using contrast agent, and the uveal signal intensity of the damaged eye was lower than the normal eye, the signal intensity at the damaged part was lower than other undamaged parts. Hirokawa *et al* [27] further studied the changes of vascular permeability in the *in vivo* rabbit eye and its duration under the effect of ultrasound combined with intravascular microbubble contrast agent. The experiment showed that the vascular permeability increased, but all the experimental rabbits did not have retinal hemorrhage, and it also showed that these changes were all transient. Yamashita *et al* [14] found that a new type of lipid microbubble + ultrasound could efficiently mediate GFP gene to be transfected into *in vivo* rat conjunctival tissue, without causing significant side effects.

**Gene therapy on ocular tumor** Deng *et al* [28] successfully cultured retinoblastoma (RB) cells *in vitro*, and the cultured cells were respectively given different ultrasonic acoustic intensity, continuous ultrasonic irradiation for 60 seconds, and microbubble contrast agent treatment of different concentrations. Thus the optimal ultrasonic acoustic intensity, irradiation time and microbubble concentration that did not significantly inhibit the activity of RB cells were screened.

According to the above conditions, EGFP gene was transfected into RB cells, and, the EGFP expression was observed after 24-48 hours under the fluorescence microscope, and finally semi-quantitative detection was conducted to mRNA of EGFP. The results showed that when the acoustic intensity  $<0.75\text{ W}/\text{cm}^2$ , irradiation for 60 seconds and microbubble concentration  $<20\%$ , the activity of RB

cells was not significantly inhibited. When the microbubble concentration was 10% and the acoustic intensity was  $0.5\text{W}/\text{cm}^2$  or  $0.75\text{W}/\text{cm}^2$ , the mediated DNA plasmid had relatively high transfection efficiency for RB cell, which was significantly higher than other experimental groups.

Zhou *et al* [29] further compared the ultrasound microbubble-mediated EGFP plasmid transfecting RB cells with the efficiency of traditional transfection methods. The results showed that the transfection efficiency of ultrasound microbubble-mediated DNA plasmid transfecting RB cells was similar with that of lipid-mediated plasmid transfection, which was significantly higher than other experimental groups. The ultrasound irradiation with certain energy and time as well as the microbubble with appropriate concentration did not significantly inhibit the activity of RB cells.

Sonoda *et al* [13] also studied the inhibitory effect of the ultrasound+microbubble plus with the anticancer drug-bleomycin for *in vivo* mouse melanoma model, and the results showed that the anticancer effect of this method was much higher than the inhibitory effect of simply giving bleomycin. Researchers believed that the duration of the cavitation effect caused by ultrasound radiation could be increased when microbubbles existed, thereby increasing the growth rate of cell membrane permeability caused by the cavitation effect. Therefore, the efficiency of transporting bleomycin to tumor cells was greatly improved.

## RESULTS

The corneal epithelial cells, RB cells and RGC were successfully transfected by the mediated target gene *in vitro*, and satisfactory transfection efficiency was obtained, thus the best transfection parameters were further selected. The findings of *in vivo* studies were also quite satisfactory, and the target gene could be successfully transfected to the rat retinal pigment epithelium through intravenous injection of ultrasound microbubble contrast agent + target gene under the ultrasound radiation with certain energy. The current studies were only in its initial stage, but the above research results proved that gene therapy using ultrasound microbubble to mediate provided new therapeutic directions for ophthalmic diseases such as RB, age-related macular degeneration, glaucoma and so on. At present, there is no specific equipment for ultrasound gene transfection, and the ultrasonic instrument, irradiation conditions and ways used by experimenters are different. Apart from different types of tissues and genes, many factors influence the microbubble contrast agent mediating gene transfection efficiency such as ultrasonic energy, irradiation time, the proportion between gene and microbubbles and so on. This technology will also produce a certain degree of harmful biological effects, and different types of contrast agents will have different gene

transfection effects. Therefore, the ultrasonic irradiation parameters must be optimized according to different contrast agents.

In recent years, the research and development of ultrasound contrast agent has achieved significant progress. On the one hand, the microbubble could finally reach the target organs and tissues through pulmonary circulation after peripheral intravenous injection because of its shortened diameter ( $<8\mu\text{m}$ ), thus the tissue echo of the target regions was strengthened. On the other hand, the stability of the microbubble of contrast agents was improved in a certain degree, and the situation of contrast agents at tissues in regions of interest could be observed. All kinds of new contrast agents like albumin, lipids and surfactants as well as those using polymer materials with promising prospects have emerged successively.

The ultrasound microbubble contrast agent is a hot research topic at home and abroad recently, having broad development prospects. With the advent of new targeting, nanoscale microbubble contrast agent and the ultrasonic instrument, it will play a huge role in the ophthalmic gene transfection.

## DISCUSSION

At present, most scholars believe that the mechanism of ultrasound microbubble is: the ultrasound microbubble contrast agent carrying genes enters into the blood through intravenous administration and reaches the target tissue through systemic blood circulation. When the ultrasonic waves of certain intensity radiate the target tissue, the tiny bubbles (cavitation nuclei) in the acoustic field rapidly expand in the negative half-cycle of the sound wave, and then they are compressed in the positive half-cycle of the sound wave to quickly collapse, forming a violent "cavitation effect". The cavitation can generate thousands of degrees of heat and hundreds of atmosphere pressures in a short time, and meanwhile phenomena such as light, shock wave, high-speed jet and so on appear, named as "sonoporation". The sound aperture can be re-closed, called "maintainable sonoporation", which can lead to the increase of cell membrane permeability. While the ultrasound microbubble contrast agent can reduce the cavitation threshold of ultrasonic waves, thereby enhancing the cavitation effect. The "shock wave" generated by the cavitation effect can increase the permeability of the local capillaries and the cell membrane of adjacent tissues, which make it easy for the target gene to get into the tissue cells, realizing and enhancing the gene transfection and expression<sup>[7-11]</sup>.

Many studies have confirmed that contrast-enhanced ultrasound has a very good safety<sup>[30,31]</sup>, but the cavitation effect of ultrasound and mechanical effect of microbubble

burst will still have harmful influences on the body tissues, and there already have been reports about contrast-enhanced ultrasound causing tissue bleeding, intravascular hemolysis and so on<sup>[32]</sup>. Besides, there are experiments indicating that the incidence of side effects such as moderate allergy, severe allergy and so on caused by contrast agents is significantly higher than previous research data<sup>[33]</sup>. So a further study is necessary for the safety of contrast-enhanced ultrasound. Secondly, whether the contrast result is good or not directly depends on the nature of contrast agents and corresponding ultrasonic parameters. As a result, the general applicability of obtained optimizing experimental conditions remains to be further observed because of different microbubble contrast agents used by various research institutions and the differences between ultrasonic instruments and radiation time.

## CONCLUSION

The research and application of contrast-enhanced ultrasound in ophthalmology is still in its initial stage, but the research findings in mediating gene transfection and targeted therapy are quite gratifying<sup>[34]</sup>. It can be predicated that contrast-enhanced ultrasound and microbubble contrast agent will play a greater role in the diagnosis and treatment of ophthalmic diseases with the continuously in-depth research on ultrasound contrast technology, constant improvement of microbubble contrast agent and the innovation of combination of molecular biology and material science combined with the ultrasound.

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