Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Medicine

journal homepage:www.elsevier.com/locate/apjtm

Document heading doi:

Enhancing effect of ultrasound-mediated microbubble destruction on gene delivery into rat kidney via different administration routes

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ARTICLE INFO

Article history:
Received 15 February 2012
Received in revised form 15 April 2012
Accepted 15 June 2012
Available online 20 July 2012

Keywords:
Ultrasound
Microbubble
Renal artery
Tail vein
Anterior tibial muscle
Renal parenchyma

ABSTRACT

Objective: To investigate the efficiency of β-galactosidase gene transfer into rat kidney with ultrasound-mediated microbubble destruction via different injection routes. **Methods:** A total of 25 Wistar rats were randomly divided into 5 groups. Four groups received a mixture of optison microbubbles (0.2 mL) and lacz plasmids (25 μg) injection via renal artery, tail vein, anterior tibial muscle and renal parenchyma, respectively. The control group received a mixture of PBS (xx mL) and lacz plasmids (25 μg) via renal artery. Three days after the gene transfer, ultrasound with fixed frequency and power (1 MHz, xxW) was delivered to the kidneys for 3 min. The efficiency of the gene transfer and expression was evaluated on the basis of β-galactosidase expression. The side effects of this method were evaluated by immunohistological method. **Results:** β-galactosidase expression could be observed only in tubules but not in glomeruli and interstitial area. The efficiency of renal artery group was higher than that of tail vein, anterior tibial muscle and renal parenchyma group ($P<0.05$). Immunohistochemical analysis revealed co-expression of β-galactosidase with a roximal tubule marker, megalin, which suggested that ultrasound enhanced gene transfer into the proximal tubular epithelial cells. No β-galactosidase expression was observed in the extrarenal organs. There were no evident pathological and biochemical changes after gene transfer. **Conclusions:** Ultrasound-mediated microbubble destruction can transfer gene into kidney via renal artery, tail vein, anterior tibial muscle and renal parenchyma. Compared with renal artery, administrating microbubbles via tail vein and anterior tibial muscle are more convenient and less vulnerarious.

1. Introduction

Renal gene transfer is a promising option for treating various genetic and acquired kidney diseases[1-3]. So far, the most widely used method for gene delivery is through viral vectors[2-5]. However, the clinical use of viral vectors is limited because of the possible disadvantages such as immunogenic properties, inflammatory responses, and the difficulty of producing large amounts of pure virus. Therefore, non-viral technologies including chemical and physical approaches have attracted scientists´ attention and are being rapidly developed. Compared with viral gene transfer, non-viral gene transfer is a very safe gene delivery method. Thus, many techniques have been employed to increase the efficacy of non-viral gene therapy recently.

Ultrasound has become an arresting technique for physically altering tissue permeability properties for improved uptake of therapeutic agents, including DNA[6]. It has many attractive features for the clinical application of renal gene therapy, such as non-invasive, highly sophisticated, flexible, cost-effective, well tolerated, and so on. Additionally, ultrasound exposure in the presence of microbubbles can achieve site-specific transfer almost anywhere in the body.

Compared with other solid organs, the kidney offers accessibility to different routes. Many researches have reported the use of renal artery injections with ultrasound exposure for the DNA delivery to the kidney[7-9]. On the other hand, there are also some reports about the other

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administration routes such as muscles injection[10,11], intravenous injection[12,13], and renal parenchymal injection[14] for the gene transfer of adeno-associated virus and other gene vectors. However, the gene transfer efficiency via different injective routes with ultrasound microbubbles aforementioned remains poorly understood. β-galactosidase gene can be expressed correctly in a variety of organisms and more easily detected and monitored with high sensitivity. Therefore, in this study we used β-galactosidase gene as a reporter gene to investigate the efficiency of ultrasound enhanced gene through four different delivery routes.

2. Materials and methods

2.1. Plasmid and ultrasound microbubble

pLenti6/v5-GW/lacZ (Invitrogen, USA), a 10 128 bp control vector expressing β-galactosidase, was amplified in *Escherichia coli*, and then isolated and purified using DNA purification kit (Promega, USA) according to the manufacturer's manual. Plasmid concentration was measured by the absorption at 260 nm with a Hitachi U-2000 (Hitachi, Japan). Optison® (Amersham GE Health Care, Princeton, NJ, USA), which has been approved by the Food and Drug Administration for the clinical applications in the USA, is a microbubble ultrasound contrast agent comprising octafluoropropane gas bodies surrounded by albumin shells. Optison of 0.2 mL 10% (v/v) concentration or phosphate buffered saline (PBS) of equivalent volume was mixed with the lacz plasmids solutions, and gently shaken by hand immediately before injection.

2.2. Experimental animal and protocol

Six-week old male Wistar rats were purchased from the academy of military medical sciences of the People's Liberation Army (PLA) and housed in the center of experimental animal, Chinese general hospital of PLA. The animals had free access to food and water. Twenty-five Wstar rats were randomly assigned into five groups: renal artery group (n=5), tail vein group (n=5), anterior tibial muscle group (n=5), renal parenchyma group (n=5), and PBS control group (n=5). In the first four groups, rats received a mixture of Optison microbubbles (0.2 mL) and lacz plasmids (25 μg) via renal artery, tail vein, anterior tibial muscle, and renal parenchyma. In the control group, rats received a mixture of PBS (0.2 mL) and lacz plasmids (25 μg) via renal artery. Immediately after the injection, an ultrasound transducer (Rich-Mar, Inola, OH, USA) was put on one side of the back close to the kidney and a continuous output of 1 MHz at 5% power was applied for a total of 60 seconds, then the transducer was put on the other side of the back close to another kidney and treated it using the same procedure. All rats were sacrificed at day 3 after gene transfer; the efficiency of gene expression was evaluated on the basis of β-galactosidase expression. To examine the possible side effects, immunohistological analysis of kidney, liver, and spleen sections were performed.

2.3. Detection of β-galactosidase expression

The rat kidney samples, which were embedded in OCT compound (Sakura, USA) and frozen in liquid nitrogen, were cut into 8 μm sections and then blown by a fan for 30 min at 20 ℃. The sections were then fixed with 2% formaldehyde plus 0.2% glutaraldehyde in PBS on ice for 20 min, washed three times in PBS for 5 min at room temperature, and then incubated in the dark 6-18 h at 37 ℃ in X-Gal solution (1 mg/mL X-Gal, 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$ in PBS, pH7.4) and counterstained with eosin for 8-10 s[5,15,16].

2.4. Immunohistochemical assay

Fresh-frozen kidney sections were fixed with 100% acetone on ice for 20 min, washed, and transferred to X-gal staining solution. The sections stained with X-gal were blocked with 1.5% H_2O_2 for 10 min, 5% bovine serum albumin/5% horse serum for 20 min, and incubated with rabbit anti-megalin (1:100, Santa Cruz, USA) over night at 4 ℃, washed with PBS for 3 times, and stained with biotin conjugated anti-rabbit IgG for 2 h at 37 ℃, and then the sections were washed with PBS for 3 times and stained with horseradish peroxidase-labeled streptavidin (1:200, Vector Laboratories, USA) for 30 min. After 3 washes with PBS, the sections were stained with 3,3′- diaminobenzidine hydrochloride.

2.5. Statistical analysis

The data were analyzed with the SPSS 15.0 statistical package and presented as mean±SD. Comparisons of characteristics among the four experiment groups were done using either one-way ANOVA or Kruskal-Wallis H test where appropriate. Statistical significance was defined as P<0.05.

3. Results

3.1. Pathological and biochemical changes in the rats after ultrasound-microbubble-mediated gene transfer via different routes

We first determined the biochemical changes in the rats after the ultrasound-microbubble-mediated gene transfer via different routes. Liver and kidney function tests showed that the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and blood

serum creatinine (Cr) in each group rats were all within the normal range. The ALT and Cr in the renal artery and renal parenchyma groups were higher than that in the PBS control group (P<0.05), while the ALT and Cr in the tail vein and anterior tibial muscle group were lower than that in the PBS control group (P<0.05). No differences in the levels of AST and BUN were found between each group (P>0.05, Table 1). Next, we used periodic acid-Schiff staining to investigate the histological changes of kidney sections from each group. Interestingly, there was neither no evident pathological change in each group after the gene transfer (Figure 1).

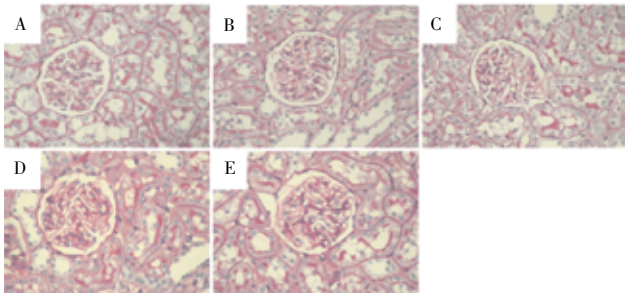

Figure 1. Renal histological changes in the rats detected by periodic acid - Schiff (PAS) staining.
No evident pathological changes were observed between each group after gene transfer. A: PAS staining of kidney sections from PBS control group. B: PAS staining of kidney sections from renal artery group. C: PAS staining of kidney sections from tail vein group. D: PAS staining of kidney sections from anterior tibial muscle group. E: PAS staining of kidney sections from anterior tibial muscle group. Magnification: ×200.

3.2. β-galactosidase expression of ultrasound-microbubble-mediated gene transfer via different routes

We next determined β-galactosidase expression in the kidneys of each group after the gene transfer via different routes. Rats from each group were injected via different routes using the procedure described in methods part and then were sacrificed 3 days after the gene delivery to evaluate β-galactosidase expression. The kidney sections from the control group showed no β-galactosidase expression (Figure 2A), whereas renal artery group, tail vein group, anterior tibial muscle group, and renal parenchyma group all showed positive β-galactosidase expression (Figure 2B, C, D, E), respectively.

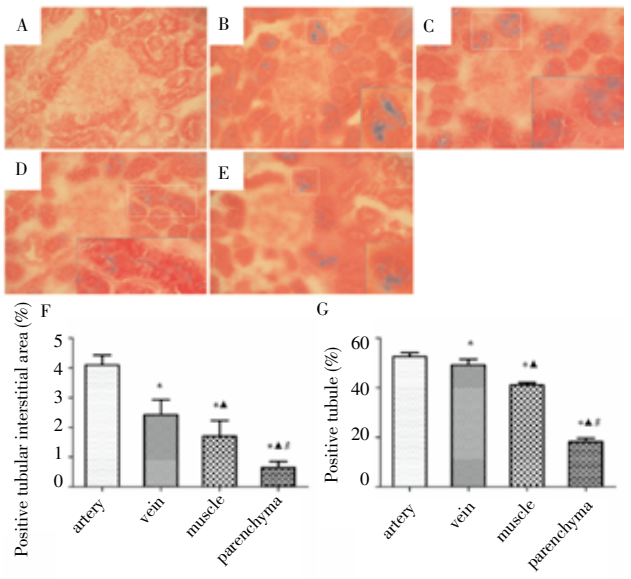

Figure 2. β-galactosidase expressions in the rats of ultrasound-microbubble-mediated gene transfer via different routes.
The rats were sacrificed on day 3, and the kidney sections were evaluated for β-gal expression. β-galactosidase expression could be observed only in tubules but not in glomeruli and interstitial area. A: β-galactosidase expression in the kidneys of PBS control group rats. B: β-galactosidase expression in the kidneys of renal artery group rats. C: β-galactosidase expression in the kidneys of tail vein group rats. D: β-galactosidase expression in the kidneys of anterior tibial muscle group rats. E: β-galactosidase expression in the kidneys of renal parenchyma group rats. Magnification, ×200. F: percentage of tubule interstitial positive area in these rats. G: percentage of positive tubules in these rats. *P <0.01 compared with renal artery group; ▲P<0.01 compared with tail vein group; #P<0.01 compared with anterior tibial muscle group, n=5.

The β-galactosidase expression was only observed in cortical tubules but not in glomeruli and interstitial area. To confirm the efficiency of gene transfer for different administration routes, the percentage of the positive area and positive tubular was measured. The results of renal artery group, tail vein group, anterior tibial muscle group, renal parenchyma group, and the control group were summarized in Table 2 and Figure 2F, G. No β-galactosidase expression was detected in the extrarenal organs (data not shown), or in tubules up to 7 days (data not shown).

3.3. Immunohistochemical analysis of megalin in kidney tissue

Using immunohistochemical assay, we found that β

Table 1.
Serum GPT, GOT, BUN and Cr levels in the rats of ultrasound-microbubble -mediated gene transfer via different routes (n=5).

Group	GPT(U/L)	GOT(U/L)	BUN(mmol/L)	Cr(μ mol/L)
PBS control	25.95±3.04	174.63±10.32	9.48±2.24	51.10±7.56
Renal artery	37.67±3.79*	177.60±16.07	10.67±2.89	65.67±6.67*
Tail vein	26.33±4.04▮	175.33±10.60	9.33±2.31	51.67±9.29▮
Anteriortibial muscle	26.67±5.69▮	177.67±15.31	10.67±1.94	54.33±5.86▮
Renal parenchyma	31.33±5.51*	178.10±30.04	11.00±2.61	59.67±7.37*

*P<0.05 compared with PBS control group; ▮P<0.05 compared with renal artery group.

-galactosidase was expressed in the rat renal cortex via renal artery injection. Megalin is a proximal tubular marker. Immunohistochemical staining revealed that both β-galactosidase and megalin expressed on the proximal tubule of the kidneys (Figure 3)[17].

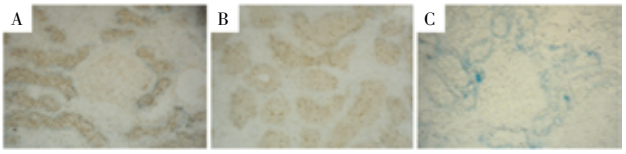

Figure 3. Immunohistochemical staining of megalin in kidney tissue. Optison microbubbles and lacz plasmids mixture were injected into the left kidney of wistar rats via renal artery, then the kidneys was exposed ultrasound for 3 min, and the rats were sacrificed on day 3, the kidney sections were evaluated for β-gal expression together with megalin immunohistochemical staining(A), megalin immunohistochemical staining(B), and β-gal expression(C).

4. Discussion

Ultrasound is thought to act by transiently permeabilizing the cell membrane, thus increasing vector uptake. The mechanism behind this effect is cavitation, the formation and oscillation of gas bubbles, in a liquid medium. Optison is an ultrasound contrast agent, used for medical imaging, consisting of albumin microspheres, filled with octafluoropropane[18,19]. Recently, Optison has been used in several sonoporation studies[20,21], in which it is thought to lower the cavitation threshold and thus enhance the effects of ultrasound on gene transfer. Compared to similar available agents, Optison was superior for US-mediated gene transfer to skeletal muscle *in vivo*[22].

It was shown that ultrasound-mediated microbubble destruction could all together enhance gene transfer into rat kidney in the four experimental groups. The efficiency of gene transferring in renal artery group rats was higher than that of tail vein group, anterior tibial muscle group and renal parenchyma group (4.10±0.33 *vs.* 2.42±0.51, 1.70±0.53, 0.65±0.20, $P<0.05$), which was similar with the adeno-associated virus[5], while the percentage of positive tubules reach up to (52.62±1.62)%, thus suggested that the transduction efficiency may consistent with clinical usage. Remarkably, only the proximal tubular epithelial cells, but not the glomeruli or vascular cells, were transduced following in our study, which means that the tubular epithelial cells are highly susceptible to ultrasound, whereas the mechanism for this phenomenon is still unclear.

Previous studies have concentrated on organs and tissues that are readily imaged by diagnostic ultrasound machines, including brain[23,24], skeletal muscle[25], heart[26,27], and kidneys[7-9]. As previous study reported, there are four different delivery routes of ultrasound and microbubbles into the kidney, such as via renal artery, via tail vein, via anterior tibial muscle, and via renal parenchyma. At present, more attention has been given to the route via ranal artery injecting[1]. We injected lacZ plasmid and Optison microbubble via these four routes above mentioned, and observed β-galactosidase expression on the proximal tubular epithelial cells of the rat kidney. Because of non-invasion, peripheral vein and skeleton muscle administration may be a potential strategy for ultrasound enhancing gene transfer, although the gene transferring efficiency of renal artery group was highest.

Plasmid mixed with Optison at the concentrations of 50% and 100% (v/v) clearly showed matrix expansion and increase in glomerular diameter[9]. On the other hand, minor inflammation[28], transient elevation in cardiac enzymes[29] and temporary AV block[30] are also reported in animal experiments. In present study, we had not observed evident pathological and biochemical changes in each group rats, which may due to the short period observation, and long-term experiments should be needed to evaluate the side effects of ultrasound enhance gene transfer.

In summary, we injected Optison microbubbles and lacz plasmids mixture into the Wistar rats via renal artery, tail vein, anterior tibial muscle and renal parenchyma, and found that β-galactosidase expressed only in proximal tubular epithelial cells but not in glomeruli and interstitial cells. The gene transfer efficiency via renal artery was higher than that of tail vein, anterior tibial muscle and renal parenchyma. Compared with renal artery, microbubbles via tail vein and anterior tibial muscle are more convenient and less vulnerarious. Peripheral vein and skeleton muscle administration may therefore be a potential strategy for ultrasound enhancing gene transfer.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

Thanks for the supports from the Second Xiangya Hospital of Central South University, and the supports from Fund from Hunan Province health science and technology support.

References

[1] Newman CM, Bettinger T. Gene therapy progress and prospects: ultrasound for gene transfer. *Gene Ther* 2007; **14**: 465-475. doi:10.1038/sj.gt.3302925

[2] Imai E, Isaka Y. Strategies of gene transfer to the kidney. *Kidney Int* 1998; **53**: 264-267.

[3] Imai E. Gene therapy approach in renal disease in the 21st century. *Nephrol Dial Transplant* 2001; **16**(Suppl 5): 26-34.

[4] Lien YH, Lai LW. Gene therapy for renal disorders. *Expert Opin Biol Ther* 2004; **4**: 919-926. doi:10.1517/14712598.4.6.919

[5] Takeda S, Takahashi M, Mizukami H, Kobayashi E, Takeuchi K, Hakamata Y, et al. Successful gene transfer using adeno-associated virus vectors into the kidney: comparison among adeno-associated virus serotype 1-5 vectors *in vitro* and *in vivo*. *Nephron Exp Nephrol* 2004; **96**: e119-e126. doi:10.1159/000077378

[6] Xie F, Boska MD, Lof J, Uberti MG, Tsutsui JM, Porter TR. Effects of transcranial ultrasound and intravenous microbubbles on blood brain barrier permeability in a large animal model. *Ultrasound Med Biol* 2008; **93**: 354-362. doi:10.1016/j.pbiomolbio.2006.07.019

[7] Azuma H, Tomita N, Kaneda Y, Koike H, Ogihara T, Katsuoka Y, et al. Transfection of NFkappaB-decoy oligodeoxynucleotides using efficient ultrasound-mediated gene transfer into donor kidneys prolonged survival of rat renal allografts. *Gene Ther* 2003; **10**: 415-425. doi:10.1038/sj.gt.3301882

[8] Lan HY, Mu W, Tomita N, Huang XR, Li JH, Zhu HJ, et al. Inhibition of renal fibrosis by gene transfer of inducible Smad7 using ultrasound-microbubble system in rat UUO model. *J Am Soc Nephrol* 2003; **14**: 1535-1548.

[9] Koike H, Tomita N, Azuma H, Taniyama Y, Yamasaki K, Kunugiza Y, et al. An efficient gene transfer method mediated by ultrasound and microbubbles into the kidney. *J Gene Med* 2005; **7**: 108-116. doi:10.1097/01.ASN.0000067632.04658.B8

[10] Rizzuto G, Cappelletti M, Mennuni C, Wiznerowicz M, DeMartis A, Maione D, et al. Gene electrotransfer results in a high-level transduction of rat skeletal muscle and corrects anemia of renal failure. *Hum Gene Ther* 2000; **11**: 1891-1900. doi:10.1002/jgm.632

[11] Maruyama H, Ataka K, Gejyo F, Higuchi N, Ito Y, Hirahara H, et al. Long-term production of erythropoietin after electroporation-mediated transfer of plasmid DNA into the muscles of normal and uremic rats. *Gene Ther* 2001; **8**: 461-468.

[12] Lien YH, Lai LW. Liposome-mediated gene transfer into the tubules. *Exp Nephrol* 1997; **5**: 132-136.

[13] Boletta A, Benigni A, Lutz J, Remuzzi G, Soria MR, Monaco L. Nonviral gene delivery to the rat kidney with polyethylenimine. *Hum Gene Ther* 1997; **8**: 1243-1251.

[14] Nakamura H, Isaka Y, Tsujie M, Rupprecht HD, Akagi Y, Ueda N, et al. Introduction of DNA enzyme for Egr-1 into tubulointerstitial fibroblasts by electroporation reduced interstitial alpha-smooth muscle actin expression and fibrosis in unilateral ureteral obstruction (UUO) rats. *Gene Ther* 2002; **9**: 495-502. doi:10.1038sjgt3301681.

[15] Miller DG, Wang PR, Petek LM, Hirata RK, Sands MS, Russell DW. Gene targeting *in vivo* by adeno-associated virus vectors. *Nat Biotechnol* 2006; **24**: 1022-1026. doi:10.1038/nbt1231

[16] Lipkowitz MS, Hanss B, Tulchin N, Wilson PD, Langer JC, Ross MD, et al. Transduction of renal cells *in vitro* and *in vivo* by adeno-associated virus gene therapy vectors. *J Am Soc Nephrol* 1999; **10**: 1908-1915.

[17] Christensen EI, Nielsen R. Role of megalin and cubilin in renal physiology and pathophysiology. *Rev Physiol Biochem Pharmacol* 2007; **158**: 1-22.

[18] Podell S, Burrascano C, Gaal M, Golec B, Maniquis J, Mehlhaff P. Physical and biochemical stability of Optisofn, an injectable ultrasound contrast agent. *Biotechnol Appl Biochem* 1999; **30**: 213-223.

[19] Kamaev PP, Hutcheson JD, Wilson ML, Prausnitz MR. Quantification of optison bubble size and lifetime during sonication dominant role of secondary cavitation bubbles causing acoustic bioeffects. *J Acoust Soc Am* 2004; **115**: 1818-1825. doi:10.1121/1.1624073.

[20] Miller DL, Song J. Tumor growth reduction and DNA transfer by cavitation-enhanced high-intensity focused ultrasound *in vivo*. *Ultrasound Med Biol* 2003; **29**: 887-893. doi:10.1016/S0301-5629(03)00031-0

[21] Taniyama Y, Tachibana K, Hiraoka K, Aoki M, Yamamoto S, Matsumot K, et al. Development of safe and efficient novel nonviral gene transfer using ultrasound: enhancement of transfection efficiency of naked plasmid DNA in skeletal muscle. *Gene Ther* 2002; **9**: 372-380. doi:10.1038sjgt3301678

[22] Li T, Tachibana K, Kuroki M, Kuroki M. Gene transfer with echo-enhanced contrast agents: comparison between Albunex, Optison, and Levovist in rats--initial results. *Radiology* 2003; **229**: 423-428. doi:10.1148/radiol.2292020500

[23] Wang JF, Wu CJ, Zhang CM, Qiu QY, Zheng M. Ultrasound-mediated microbubble destruction facilitates gene transfection in rat C6 glioma cells. *Mol Biol Rep* 2008; 12. doi: 10.1007/s11033-008-9307-3

[24] Takahashi M, Kido K, Aoi A, Furukawa H, Ono M, Kodama T. Spinal gene transfer using ultrasound and microbubbles. *J Control Release* 2007; **117**: 267-272. doi:10.1016/j.jconrel.2006.10.026

[25] Lu QL, Liang HD, Partridge T, Blomley MJ. Microbubble ultrasound improves the efficiency of gene transduction in skeletal muscle *in vivo* with reduced tissue damage. *Gene Ther* 2003; **10**: 396-405. doi:10.1038/sj.gt.3301913

[26] Müller OJ, Schinkel S, Kleinschmidt JA, Katus HA, Bekeredjian R. Augmentation of AAV-mediated cardiac gene transfer after systemic administration in adult rats. *Gene Ther* 2008; **10**: 1-8. doi:10.1038/gt.2008.111

[27] Mayer CR, Bekeredjian R. Ultrasonic gene and drug delivery to the cardiovascular system. *Adv Drug Deliv Rev* 2008; **60**: 1177-1192. doi:10.1016/j.addr.2008.03.004

[28] Pislaru SV, Pislaru C, Kinnick RR, Singh R, Gulati R, Greenleaf JF, et al. Optimization of ultrasound-mediated gene transfer: comparison of contrast agents and ultrasound modalities. *Eur Heart J* 2003; **24**: 1690-1698. doi:10.1016/S0195-668X(03)00469-X

[29] Guo DP, Li XY, Sun P, Wang ZG, Chen XY, Chen Q, et al. Ultrasound/microbubble enhances foreign gene expression in ECV304 cells and murine myocardium. *Acta Biochim Biophys Sin (Shanghai)* 2004; **36**: 824-831.

[30] Tsunoda S, Mazda O, Oda Y, Akabame S, Kishida T, Shin-Ya M, et al. Sonoporation using microbubble BR14 promotes pDNA/siRNA transduction to murine heart. *Biochem Biophys Res Commun* 2005; **336**: 118-127. doi:10.1016/j.bbrc.2005.08.052