Ultrasound-targeted microbubble destruction enhances AAV mediated gene transfection: human RPE cells in vitro and the rat retina in vivo

Hongli Li[1,3], Xiaozhi Zheng[1,3], Huiping Wang[2], Fan Li[1], Ying Wu[1] and Lianfang Du[1]

[1] Department of Ultrasound, Shanghai Jiaotong University Affiliated First People's Hospital, 85 Wu Jin RD, 200080 Shanghai, China; [2]Experimental Research Center of Shanghai Jiaotong University Affiliated First People's Hospital, 85 Wu Jin RD, 200080 Shanghai, China.

Correspondence: Dr Lianfang Du, Department of Ultrasound, Shanghai Jiaotong University Affiliated First People's Hospital, 85 Wu Jin RD, 200080 Shanghai, China.

E-mail: Du_lf@163.com

3 –These authors contributed equally to this work.

Abstract

The present study was performed to investigate the efficacy and safety of Ultrasound-targeted microbubble destruction (UTMD) mediated rAAV2-EGFP to cultured human retinal pigment epithelium (RPE) cells in vitro and the rat retina in vivo. In vitro study, cultured human RPE cells were exposed to US under different conditions with or without microbubbles. Furthermore, the effect of UTMD to rAAV2-EGFP itself and the cells were evaluated. In vivo study, gene transfer was examined by injecting rAAV2-EGFP into the subretinal space of the rats with or without microbubbles and then exposed to US. We investigated EGFP expression in vivo via stereomicroscopy and performed quantitative analysis by Axiovision 3.1 software. HE staining and frozen sections were used to observe tissue damage and location of EGFP gene expression. In vitro study, the transfection efficiency of rAAV2-EGFP increased 74.85% under the optimal UTMD conditions. Furthermore, there was almost no cytotoxicity to the cells and rAAV2-EGFP itself. In vivo study, UTMD could be used safely to enhance and accelerate transgene expression of the retina. Fluorescence expression was mainly located in the layer of retina. UTMD is a promising method for gene delivery to the retina.

【Key words】Ultrasound; Microbubbles; AAV; human RPE cells; Retina; Gene transfection

Introduction

Many inherited and acquired retinal diseases have become the leading causes of blindness in adults and RPE cells are believed to be involved in the pathogenesis of some such diseases, such as retinitis pigmentosa (RP), Leber Congenital Amaurosis (LCA), and proliferative vitreoretinopathy (PVR).[1,2] Currently, there is no satisfactory treatment available for these disorders. Gene therapy represents a

promising therapeutic option for these diseases. One of the most critical areas of gene therapy is the design of an appropriate, accurate, and effective gene transfer system that can be safely applied in vivo. Adeno-associated virus (AAV) vectors have a number of important advantages over other vectors which make them suitable for transfection studies, in particular the ability to induce long-term transgene expression in the eye and a relative lack of pathogenicity.[1,3,4] However, the transduction of AAV occurs with relatively low efficiency which limits its therapeutic effects. Maybe increasing AAV transduction could produce better therapeutic effects.[5-7]

UTMD has been proved to enhance gene transfer in various studies in vitro and in vivo. UTMD could serve as a potential site-specific gene transfer modality, but the mechanism is not definite. Sonoporation which can transiently enhance the permeability of cell membranes to introduce genes into cells by exposing them to ultrasound is considered to play a key role, and microbubbles acted as cavitation nuclei can effectively focus ultrasound energy and further potentiate bioeffects.[8-11] Recent reports show that Ultrasound (US) / Microbubbles (MBs) can be used safely and efficiently in many models of diseases in various organs.[12] As far as we know, in the sphere of ophthalmology, scholars pay much attention to studying ultrasound-mediated transfection (USMT) or ultrasound-targeted microbubble destruction (UTMD) on the cornea,[13-17] but few reports on the retina. Moreover, ultrasound/ microbubbles mediated transduction mainly focuses on plasmid-DNA, few reports have been published about ultrasound/ microbubbles and AAV. Thus we got the idea that since UTMD could greatly increase plasmid-DNA transfer to the corneal cells in vivo and in vitro, whether UTMD could increase rAAV2-EGFP transfection to the retina.

Results

In vitro study

1. Gene transfer by ultrasound alone

The US intensities and the exposure time were set at 1 W/cm^2, 2 W/cm^2, 3 W/cm^2, 60s and 120s respectively (50% duty cycle). There was no difference between the US exposure groups and the control group (P>0.05, Figure 1A).

2. Gene transfer by microbubbles alone

The ratios of MBs to cells we selected for our study were 40:1 and 50:1. There was no difference between the groups in which the cells were treated with microbubbles alone and the control group (P>0.05, Figure 1B).

3. Gene transfer by ultrasound and microbubbles

To identify the optimal conditions to transfer rAAV2-EGFP by ultrasound with microbubbles, the following four parameters were examined.

3.1 Pulse wave and continuous wave

Pulse wave and continuous wave were examined under an intensity of 2 W/cm^2 for 60s with microbubbles (the ratio of MBs to cells as 50:1). Three duty cycles (10%, 20%, 50%) of pulse wave and continuous wave were studied. As a result, the ratios of the EGFP-positive cells were 17.68% of the control group, 17.53%, 25.77%, 30.43% with 10%, 20%, 50% duty cycle respectively and 33.67% with continuous wave. Under the condition of 50% duty cycle, UTMD could significantly enhance the transfection efficiency (Figure 2A, P<0.05). Although UTMD could significantly enhance the transfection efficiency under the condition of

continuous wave (P<0.001), the cell viability was lower than 85.0% (Figure 2C).

3.2 Microbubbles

Five ratios of MBs to cells (20:1, 30:1, 40:1, 50:1, 60:1) were examined under the US condition of 1 W/cm^2, 60s, and 50% duty cycle. The increased percentage of transfection efficiency in 50:1 group was significantly than that in 20:1, 30:1 and 40:1 groups (P<0.05, Figure 3A). There was no difference between the ratios of 50:1and 60:1 (P>0.05).

3.3 US Intensity and Exposure Time

The US intensities of 1 W/cm^2, 2 W/cm^2, 3 W/cm^2 and the exposure time of 60s and 120s were examined (50% duty cycle and the ratio of MBs to cells as 50:1). The ratio of EGFP-positive cells in the UTMD (US+MBs) group was significantly higher than that of the corresponding USMT (US) group (P<0.01, Figure 3B). In the UTMD group, there was no difference between the 1 W/cm^2, 60s and other conditions (P>0.05, Figure 3B). Moreover, under the condition of 1 W/cm^2 and 60s, little cell damage was observed by Trypan Blue assay.

4. The effect of UTMD to rAAV2-EGFP

The effect of UTMD to rAAV2-EGFP itself was evaluated by the transfection efficiency of the cells after UTMD. There was no difference between the experimental groups (power, 1 W/cm^2 and 3 W/cm^2; 50% duty cycle; 60s and 120s; the ratio of MBs to cells as 50:1) and the control group (P>0.05, Figure 4).

5. Cell viability assay

We performed Trypan Blue assay immediately after UTMD with various exposure parameters to measure

the cytotoxicity of the cells. Under the condition of 1 W/cm^2, 2 W/cm^2, 3 W/cm^2, 50% duty cycle, 120s, the ratio of MBs to cells as 50:1, the cell viability decreased in sequence. Under the condition of 3W/cm^2, 120s, and 60:1, the cell viability decreased to 58.67% (Figure 5).

In vivo study

1. The number of EGFP positive rats on the 4th day: The number of EGFP positive rats of Group 4 was significantly more than that of other groups (P<0.05, Table 1).
2. EGFP expressions of four groups at different time points. The quantity of fluorescence in Group 4, in which AAV was combined with ultrasound and microbubbles, was significantly higher than that in other three groups on the 4th, 7th and 35th day. There was no difference for the longer days. EGFP expression increased from the 7th to the 35th day, and fell from the 49th to the 120th day (Figure 6).
3. Tissue stretched preparation on the 35th day. The number of transfected cells in Group 4 (Figure 7B) was more than that in Group 1 (Figure 7A) and EGFP expression mainly showed in RPE cells (Figure 7C).
4. Histology showed no evident tissue damage after UTMD mediated AAV transfection (Figure 8).
5. The frozen section of fundus oculi showed that EGFP expression mainly appeared in the layer of retina (Figure 9).

Table 1 The number of EGFP positive rats on the fourth day

Group/ Number	Group1	Group 2	Group 3	Group 4
Number of EGFP positive rats	5	4	5	10
Number of observed rats	12	12	11	11

χ^2 = 9.051, P<0.05, The number of EGFP positive rats in Group 4 was significantly more than that in other groups.

Discussion

Gene therapy can potentially correct inherited and acquired diseases of the retina. The retina has structure and accessibility properties which make it an ideal target organ for genetic therapies. Moreover, in the last two decades, over 130 genes have been identified to cause retinal diseases and many animal models also have been built.[1,3,18-21] Recently, the potential for gene delivery to the eye using AAV vectors has received much attention.[1,3,4] However, the transfection of AAV occurs with relatively low efficiency and later expression in some tissues, which limits its therapeutic effects. Several methods to increase the transfection efficiency of AAV vectors have been explored, such as combinational use with adenovirus or drug of chemotherapy,[6] However, the above strategies have many limitations. We used sodium butyrate combined with rAAV2-EGFP to transfect human RPE cells in vitro. We achieved higher transfection efficiency, but higher cell death rate (data not shown). A new and improved method of delivery system is required to augment the efficiency of AAV infection.

Ultrasound/microbubbles associated local gene therapy has potential for not only plasmid-DNA, but also virus-mediated gene transfer, just like adenovirus, adeno-associated virus, retroviral.[22-27] Ralph V. Shohet et al demonstrated successful transfection of rat myocardium in vivo by UTMD of microbubbles containing an adenovirus encoding a beta-galactosidase reporter gene.[23] Transfection efficiencies by ultrasound with retrovirus were enhanced 6.6-fold, 4.8-fold, 2.3-fold, and 3.2-fold in 293T cells, BAECs, RASMCs, and L6 cells, respectively.[25] Recent research outcomes showed that UTMD could augment cardiac transfection of

AAV vectors after intravenous administration in rats. UTMD significantly increased cardiac reporter activities 6-20 fold compared with control rats which received the same amount of virus without microbubbles, but with ultrasound.[22]

Our findings indicated that UTMD could exert a significant enhancing effect on rAAV2-mediated gene expression in RPE cells. To establish the optimal conditions of UTMD mediated gene transfer to human RPE cells, various US conditions were examined. First, the duty cycle of US was evaluated. Results clearly showed that a continuous wave was most effective to transfer genes, but cell damage was severe. Duty cycles of 10% and 20% provided almost identical gene transfer efficiency. A duty cycle of 50% increased transfection efficiency significantly. Therefore, a duty cycle of 50% was chosen for our purpose. Second, the ratio of MBs to cells was evaluated. A ratio of 20:1 could not make the gene transfer efficiently and the ratios of 30:1and 40:1 provided almost identical gene transfer efficiency. There was no difference between the ratios of 50:1and 60:1. Thus, 50:1 was chosen. Third, US power and exposure time were studied. Comprehensively considering gene transfer efficiency and cellular damage, an US power of $1W/cm^2$ was chosen, and exposure time was set at 60s. Finally, rAAV2-EGFP transfection efficiency after UTMD was evaluated. There was no difference between the exposure groups at varying power levels ($1w/cm^2$, 60s; $3w/cm^2$, 60s; $1w/cm^2$, 120s; $3w/cm^2$, 120s) and the control group. That is to say UTMD had no apparent effect to rAAV2-EGFP transfection efficiency. The ratios of EGFP-positive cells in the above exposure groups were lower than that of the control group, because there were some viruses losing when we transferred them from the exposure well to the wells with cells.

Our studies showed that AAV combined with microbubbles without ultrasound or with ultrasound exposure

alone could not increase the transfection efficiency, but UTMD could increase rAAV2 transfer to human RPE cells in vitro. The optimal parameters were as follows: power, 1 W/cm^2; duration, 60s; duty cycle, 50%; the ratio of MBs to cells as 50:1. Under this condition, UTMD increased the transfection efficiency about 74.85% , and the cell viability was above 95%. Furthermore, there was almost no cytotoxicity to rAAV2-EGFP itself.

Our previous study used methyl thiazolyl tetrazolium (MTT) assay to measure human RPE proliferation after UTMD for 24 hours and 72 hours.[28,29] In this study, we used Trypan Blue assay to measure the cell viability immediately after UTMD. The optimal parameters combination we filtrated was safe (The cell viability was higher than 95%) not only immediately but also 24 and 72 hours later after UTMD. Our team members achieved the similar trends of UTMD mediated rAAV2-EGFP transfection in human renal carcinoma cells in vitro and demonstrated that UTMD mediated gene delivery occurred principally via enhancing gene entry rather than the transcriptional level or DNA replication (data not shown). Much of reports showed that USMT could enhance gene transfer efficiency, but in our study, USMT did nothing effective to human RPE cells. Maybe higher energy was needed for transient pore of human RPE cells.

In vivo study, scholars pay much attention to studying USMT on the cornea. In the beginning, they focused on drug delivery.[14] Recently, gene delivery of the cornea has been studied. There was a study showed that using US in conjunction with commercially available MBs could enhance gene delivery to the cornea without damaging tissues.[16] Saito K et al found that the degree of cell injury induced by ultrasound scaled with exposure intensity and duration.[17] Our previous study showed that ultrasound combined with Sonovue could increase plasmid transfer to the mouse cornea in vivo safely.[30]

In our preliminary experiment in vivo, we used different grouping of power (1 w/cm^2, 2 w/cm^2, 3 w/cm^2) and MBs (1, 2μl) with duration of 5 minutes. We selected 2w/cm^2 and 1μl MBs as our experimental conditions. Just like our study in vitro, USMT and MBs alone did nothing effective to the transfection efficiency of the retina in vivo. UTMD could make rAAV2-mediated EGFP express earlier after injection and substantially increase gene expression before the fastigium (35days). The difference was smaller gradually along with the time prolonging. Although the transfection efficiency of Group 4 was higher than that of other groups, there was no difference between them after 35days. The mechanism by which US enhanced gene delivery was thought to be linked to sonoporation, and UTMD could intensify the bioeffect of sonoporation. Sonoporation provided another way for AAV entering the cells, and the transient pores appeared immediately after US exposure, so AAV entered the cells by UTMD earlier and much more than by natural infection. The fact that UTMD enhanced rAAV2-mediated gene expression in the live retina was further confirmed by fluorescent stereoscope imaging of the rat retina and fluorescence quantitative methods by Axiovision 3.1 software. Integrated optical density (IOD) values showed that UTMD (Group 4) enhanced expression levels about 82.12%, 44.58%, 27.1% and 21.9% on the 4th, 35th, 49th and 120th day respectively compared with natural infection (Group 1). These data strongly suggested the potential utility of UTMD for improving expression levels of r AAV2-mediated gene transfer in the retina in vivo.

In our study, EGFP expression trends of the four groups were similar to previous reports.[31,32] Frozen sections of fundus oculi showed EGFP expression major in the layer of retina. The hexagonal shapes of the fluorescent cells were clearly identified at high magnification (×400), suggesting that the majority of the transfected cells were RPE cells. This was similar to previous reports. [31,32]

The novelty of our study was that our report described the first successful demonstration that UTMD could be used not only to enhance, but also to accelerate transgene expression of the retina. UTMD provided a new way for systemic administration of AAV vectors.

In this study, we approved that UTMD could enhance AAV transfection efficiency in human RPE cells in vitro and Wistar rat retina in vivo. How to enhance the transfection efficiency furthest and how to maximize the amount of AAV that can be attached to MBs are worthy to be studied in future. We only evaluated the tissue structure damage of the retina, further evaluation of vision threaten is much more important, especially for eventual clinical application.

Materials and methods

In vitro study

Cell Culture

ARPE-19 (human retinal pigmented epithelium) cell line was obtained from American Type Culture Collection (CRL-2302™, ATCC, Rockville, Maryland, USA) and the cells were incubated in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum in a 37 °C, 5% CO_2/95% air according environment. Human RPE cells were put into 24-well plates every other well at 2×10^5 cells/well prior to infection.

Virus

The type 2 recombinant adeno-associated viral vectors encoding enhanced green fluorescent protein gene

(rAAV2-CMV-EGFP) was purchased from Vector Gene Technology Company Limited (Beijing, China).

MBs

SonoVue (Bracco, Milan, Italy), a lipid-shelled US contrast agent filled with sulfur hexafluoride gas, is composed of about 2×10^8 /ml microbubbles, having an average diameter of 2.5–6.0 μm.

Ultrasound Exposure Protocol

A therapeutic ultrasound machine (Topteam161, Chattanooga, America) was used. The area of the probe was 25 mm^2. The cells were exposed to ultrasound (frequency, 1 MHz; power, 1 W/cm^2, 2 W/cm^2, 3W/cm^2; duration, 60 and 120seconds; pulse wave with 10%, 20%, 50% duty cycle and continuous wave; pulse recurrent frequency, 100Hz) with or without MBs. The dosage of SonoVue was selected according to the ratio of MBs to cells (20:1-60:1). The ultrasound transducer was placed on the bottom of the plates with a small amount of coupling medium on the surface of the probe. A self-made plastic disc with a hole in the middle was placed between the transducer and the bottom of the plates. The purpose of using the self-made plastic disc was to avoid interaction among the radiated wells as well as to ensure the same thickness of coupling medium between the probe and the plate. The DMEM volume per well was 150μl when exposed to US. To attach rAAV2-EGFP to microbubbles sufficiently, rAAV2-EGFP was mixed with microbubbles in equivalent volume, and the mixture was incubated at room temperature for 5 minutes to allow an electrostatic attachment. Then the mixed solution was added to the plates and exposed to US as described. 2 hours after infection, the plates were supplemented with DMEM to final volume 500μl. 12 hours after infection, the previous DMEM was replaced by fresh DMEM.

Gene transfer efficiency

48 hours after transfection, EGFP expression was observed and photographed via inverted fluorescence microscopy (ZEISS Axiovert S 100, Jena, Germany). The ratio of the infected cells was examined by FACS (EPICS XL, Beckman Coulter Company, Miami, FL, USA).

Cell viability assay

We performed Trypan Blue assay immediately after UTMD with various exposure parameters to measure the cytotoxicity of the cells. The cells were then analyzed to determine the proportion of positive blue stained cells through microscopy.

The effect of UTMD to rAAV2-EGFP

The effect of UTMD to rAAV2-EGFP itself was evaluated by the transfection efficiency to the cells after UTMD. After 150μl medium containing rAAV2-EGFP only was exposed to varying conditions (power, 1 W/cm^2 and 3 W/cm^2; 50% duty cycle; 60s and 120s; the ratio of MBs to cells as 50:1), the cells were transfected by the medium and then the transfection efficiency was evaluated.

In vivo study

Animal preparation

Normal adult Wistar rats (180–200g) were used in this experiment. All animals were treated, maintained, and sacrificed in accordance with the policies stated in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines approved by national and local institutions. The rats were anesthetized with intraperitoneal injection of 10% chloral hydrate. Pupillary dilatation was achieved by using Tropicamide eyedrops. The eyes were gently protruded by a plastic circle and subsequently

covered with Ofloxacin Eye Ointemnt. A 26 gauge needle was inserted 1 mm posterior to the corneal limbus causing a self-sealing wound tunnel under surgical microscopy (SM-2000J, Shanghai, China). Trans-sclera subretinal injection of 3μl viral suspension was made by introducing a micro-injector through the sclera and vitreous into subretinal space. The eyes of rats were injected with 3μl rAAV2-EGFP alone or combined with 1μl microbubbles or 1μl normal saline (NS). The doses of viral vectors were 3×10^9 vg /eye.

Animal grouping

78 rats were used in this study. The rats were divided into 4 groups as follows:

Group 1: AAV and NS without microbubbles and ultrasound exposure

Group 2: AAV and MBs without ultrasound exposure

Group 3: AAV and NS with ultrasound exposure but without microbubbles

Group 4: AAV and MBs with ultrasound exposure (experimental group)

In each group, at least 5 rats survived for more than 4 months. Group 4 was the experimental target and the remaining groups served as controls. Due to the risk of an overload of volume, we administered the infusions (4μl) slowly.

Ultrasound Exposure Protocol

Right after subretinal injection, the eyes of Group 3 and Group 4 were exposed to ultrasound. The frequency was 1MHz, US power was $2w/cm^2$ and the pulse repetition frequency was 100Hz with 50% duty cycle. The entire treatment lasted for 5 minutes.

EGFP Expression

The presence of fluorescence signaling in the in vivo gene expression was determined by direct stereomicroscopy (ZEISS, Stemi SV11, Jena, German) on the 4^{th}, 7^{th}, 35^{th}, 49^{th}, and 120^{th} day after gene transfer. We investigated the onset of EGFP gene expression, lightness of fluorescence, area of fluorescence and its distribution in the fundus in vivo. The number of EGFP positive rats on the 4^{th} day was evaluated by two masked observers. The value of gene transfer was quantified through EGFP fluorescence quantitative methods by Axiovision 3.1 software. IOD = eye area (μm^2) × mean fluorescence intensity (grey).

Histology

Four eyes under US exposure ($2w/cm^2$, 1μl microbubbles, 5 minutes) were obtained by overdose intraperitoneal injection of 10% chloral hydrade to four rats on the 4^{th} day after subretinal injection. The sections were stained with hematoxylin and eosin to observe retinal architecture by microscopy (ZEISS, Axioplan 2 Imaging, Jena, Germany). All the specimens were observed by two masked observers who received no information about the specimens.

Tissue stretched preparation of fundus oculi

Two eyes were harvested from each group on the 35^{th} day. We visualized the transfected tissue by inverted fluorescent microscopy on flat fundus oculi. Fundus oculi were prepared after enucleation of the globe by removing the anterior segment with a blade and carefully transferring the whole fundus oculi to a microscope slide. Six relieving incisions were made to allow the retina to be flattened. The tissue was sealed with glycerin.

Frozen sections of eyeballs

Two eyes were harvested from each group on the 35th day postinjection. The enucleated eyes were embedded in O.C.T. compound (Sakura, America) and cryosections (10 μm) were cut by cryotome (Leica, CM3050S, Germany). We observed the cryosections by fluorescence microscopy (ZEISS, Axioplan 2 Imaging, Jena, Germany).

Statistical Analysis

All values were expressed as means and standard deviations (mean ± SD). ANOVA test was used to determine the significance of the differences in multiple comparisons. Chi-Square Test was used to determine the significance of the measurement data. A value of $P<0.05$ was considered to be statistically significant. The software packages used were SPSS, version 13 (SPSS 13.0, Chicago).

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References

1 Enrico M. Surace, Alberto Auricchio. Versatility of AAV vectors for retinal gene transfer. *Vision*

Research 2008; **48**: 353–359.

2 Li Zhang, Xiaoxin Li, Mingwei Zhao, Peiying He, Weidong Yu, Jianqiang Dong, et al. Antisense oligonucleotide targeting c-fos mRNA limits retinal pigment epithelial cell proliferation; a key step in the progression of proliferative vitreo-retinopathy. Experimental *Eye Research* 2006; **83**: 1405-1411.

3 Keith R.G. Martin, Ronald L. Klein, Harry A. Quigley. Gene delivery to the eye using adeno-associated viral vectors, *Methods* 2002; **28**: 267–275.

4 Astra dinculescu, Lyudmyla Glushakova, Seok-hong Min, William W.Hauswirth. Adeno-associated viral vectors for retinal gene transfer. *Human Gene Terapy* 2005; **16**: 649–663.

5 Zhang, S. H., Wu, J. H., Wu, X. B., Dong, X. Y., Liu, X. J., Li, C. Y., et al. Distinctive gene transduction efficiencies of commonly used viral vectors in the retina. *Current Eye Research* 2008; **33**: 81-90.

6 Jihong Wu, Shenghai Zhang, Xiaobing Wu, Xiaoyan Dong, Ping Xu, Xinjian Liu, et al. Enhanced transduction and improved photoreceptor survival of retinal degeneration by the combinatorial use of rAAV2 with a lower dose of adenovirus. *Vision Research* 2008; **48**: 1648-1654.

7 WU Jihong, ZHANG Shenghai, LIU Yan, CHEN Xiafang, XU Ping, WU Xiaobing, et al. Transduction efficacy of recombinant type 1 and type 2 adeno-associated virus in the retinal cells. *NatlMed J China* 2006; **86**: 2841-2846.

8 Gail ter Haar. Therapeutic applications of microbubbles. *Progress in Biophysics and Molecular Biology* 2007; **93**: 111-129.

9 Evan C. Unger, Terry Onichi Matsunaga, Thomas McCreery, Patricia Schumann, Robert Sweitzer, Rachel Quigley. Therapeutic applications of microbubbles. *European Journal of Radiology* 2002; **42**:160-168.

10 P.A. Dijkmans, L.J.M. Juffermans, R.J.P. Musters, A. van Wamel, F.J. ten Cate, W. van Gilst, etal. Microbubbles and ultrasound: from diagnosis to therapy. *Eur J Echocardiography* 2004; **5**: 245-256.

11 Kengo OKADA, Nobuki KUDO, Koichi NIWA, Katsuyuki YAMAMOTO. A basic study on sonoporation with microbubbles exposed to pulsed ultrasound. *J Med Ultrasonics* 2005; **32**: 3–11.

12 Tetsuo NOZAKI, Ryohei OGAWA, Akihiko WATANABE, Remon NISHIO, Hideki FUSE, Takashi KONDO. Ultrasound-mediated gene transfection: problems to be solved and future possibilities. *J Med Ultrasonics* 2006; **33**: 135–142.

13 Vesna Zderic, John I. Clark, Roy W. Martin, Shahram Vaezy. Ultrasound-enhanced transcorneal drug delivery. *Cornea* 2004; **23**: 804-811.

14 Zderic V, Clark JI, Vaezy S. Drug delivery into the eye with the use of ultrasound. *J Ultrasound Med* 2004; **23**:1349-1359.

15 Vesna Zderic, Shahram Vaezy, Roy W. Martin, John I. Clark, Ocular drug delivery using 20-kHz ultrasound, *Ultrasound in Med. & Biol* 2002; **28**: 823–829.

16 Shozo Sonoda, Katsuro Tachibana, Eisuke Uchino, Akiko Okubo, Matsuo Yamamoto, Kenji Sakoda, et al. Gene transfer to corneal epithelium and keratocytes mediated by ultrasound with microbubbles. *IOVS* 2006; **47**: 558-564.

17 Kiyofumi Saito, Katsuya Miyake, Paul L. Mcneil, Keiichiro Kato, Keiko Yago Naonori Sugai. Plasma membrane disruption underlies injury of the corneal endothelium by ultrasound. *Exp. Eye Res* 1999; **68**: 431-437.

18 Ji-jing Pang, Amanda Lauramore, Wen-tao Deng, Qiuhong Li, Thomas J. Doyle,Vince Chiodo, et al. Comparative analysis of in vivo and in vitro AAV vector transduction in the neonatal mouse retina: Effects of serotype and site of administration. *Vision Research* 2008; **48**: 377-385.

19 Bennett J. Gene therapy for retinitis pigmentosa. *Curr Opin Mol Ther* 2002; **2**: 420-425.

20 Jean Bennett, Albert M. Maguire. Gene therapy for ocular disease. *Mol Ther* 2000; **1**: 501-505.

21 F Rolling. Recombinant AAV-mediated gene transfer to the retina: gene therapy perspectives. *Gene Therapy* 2004; **11**: 26–32.

22 O J Müller, S Schinkel, J A Kleinschmidt, H A Katus, R Bekeredjian. Augmentation of AAV-mediated cardiac gene transfer after systemic administration in adult rats. *Gene Therapy* 2008; **15**: 1558–1565.

23 Ralph V. Shohet, Shuyuan Chen, Yan-Ting Zhou, Zhuowei Wang, Robert S. Meidell, Roger H. Unger, et al. Echocardiographic Destruction of Albumin Microbubbles Directs Gene Delivery to the Myocardium. *Circulation* 2000; **101**: 2554-2556.

24 Sarah L. Taylor, Ahad A. Rahim, Nigel L. Bush, Jeffrey C. Bamber, Colin D. Porter. Targeted retroviral gene delivery using ultrasound. *J Gene Med* 2007; **9**: 77–87.

25 Toshio Naka, Tsuyoshi Sakoda, Takashi Doi, Takeshi Tsujino, Tohru Masuyama, Seinosuke Kawashima, et al. Ultrasound Enhances Retrovirus-Mediated Gene Transfer. *Preparative Biochemistry and Biotechnology* 2007; **37**: 87-99.

26 Shuyuan Chen, Ralph V. Shohet, Raffi Bekeredjian, Peter Frenkel, Paul A. Grayburn. Optimization of Ultrasound Parameters for Cardiac Gene Delivery of Adenoviral or Plasmid Deoxyribonucleic Acid by Ultrasound-Targeted Microbubble Destruction. *Journal of the American College of Cardiology* 2003; **42**: 301-308.

27 Candace M. Howard, Flemiming Forsberg, Corrado Minimo, Ji-Bin Liu, Daniel A. Merton, Pier Paolo Claudio. Ultrasound Guided Site Specific Gene Delivery System Using Adenoviral Vectors and Commercial Ultrasound Contrast Agents. *J. Cell. Physiol* 2006; **209**: 413–421.

28 Wu Ying, Du Lianfang, Gu Qing. Impact of Ultrasound Exposure Combined with SonoVue on Retinal Pigment Epithelium Proliferation. *Chinese J Ultrasound Med* 2007; **123**: 561-563.

29 Wu Ying, Du Lian fang, Gu Qing, Wang Huiping, Wang Feng, Li Fan. Optimal irradiation conditions of SonoVue combined with ultra sound mediated gene transfection to retinal pigment epithelium: vitro study. *Chin J Med Ultrasound (Electronic Version)* 2007; **4**: 334-337.

30 Wu Ying, Du Lianfang, Chen Yongdong, Wang Huiping, Wang Feng. SonoVue and ultrasound mediated pEGFP-N1 transfection to mouse cornea in vivo study. *Chinese Journal of Ultrasonography* 2008; **17**: 350-353.

31 Fabienne Rolling, Wei-Yong Shen, Hyacinthtabarias, Ian Constable,Yogesan Kanagasingam, Chris J. barry, et al. Evaluation of Adeno-Associated Virus-Mediated Gene Transfer into the Rat Retina by Clinical Fluorescence Photography. *Human Gene Therapy* 1999; **10**: 641- 648.

32 Fabienne Rolling, Wei-Yong Shen, Nigel L Barnett, Hyacinth Tabarias, Yogesan Kanagasingam, Ian Constable FRACO, et al. Long-term real-time monitoring of adeno-associated virus-mediated gene expression in the rat retina. *Clinical and Experimental Ophthalmology* 2000; **28**: 382–386.

Figure 1 Gene transfer by ultrasound or microbubbles alone. (A) There was no difference between the control group and the ultrasound exposure groups (P>0.05). (B) There was no difference between the control group and the microbubbles groups (P>0.05).

Figure 2 The ratios of EGFP-positive cells under pulse wave and continuous wave (the ratio of MBs to cells as 50:1). (A) Under the condition of 50% duty cycle, UTMD could significantly enhance the transfection efficiency (P<0.05). (C) Apparent cell damage was found in many cells (arrows) and the distribution of cells was sparse under the condition of 2w/cm^2 and continuous wave.

Figure 3 Gene transfer by US and MBs under different conditions. (A) The increased percentage of transfection efficiency in 50:1 group was significantly higher than that in 20:1, 30:1 and 40:1 groups (P<0.05). (B) The ratios of EGFP-positive cells in UTMD (US+MBs) group was significantly higher than that of USMT (US) group (P<0.01). In UTMD group, there was no difference between the 1 W/cm^2, 60s group and other groups (P>0.05).

Figure 4 The effect of UTMD to rAAV2-EGFP itself . There was no difference between the experimental groups and the control group (P>0.05).

Figure 5 Cell damage under different US intensities and exposure time. With the increase of irradiation intensity, the cell viability decreased gradually.

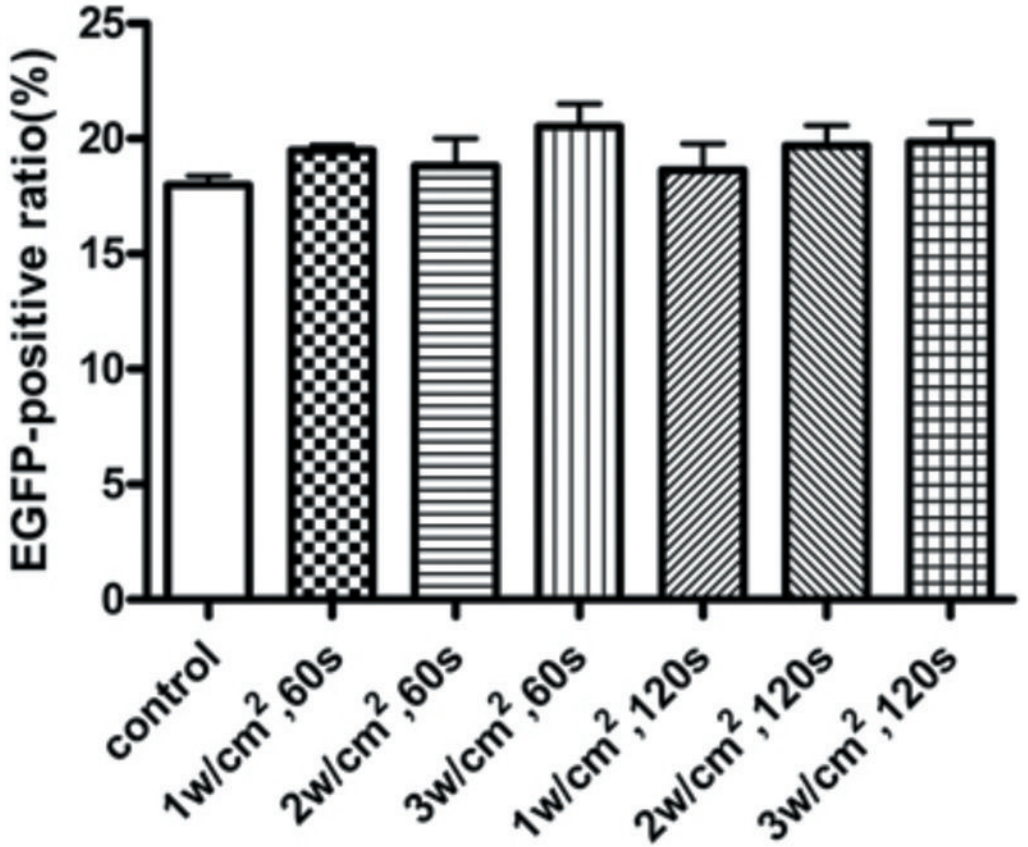
Figure 6 (A) rAAV2-EGFP expression after subretinal injection on the 4^{th}, 7^{th} and 35^{th} day by direct fluorescence stereomicroscopy ×25. rAAV2-EGFP expression of Group 4 was visible on the 4^{th} day after subretinal injection, and there was no rAAV2- EGFP expression in other groups. (B) EGFP expression increased from the 7^{th} to the 35^{th} day, and fell from the 49^{th} day to the 120^{th} day. (C) EGFP expression of the fourth Group 4 was significantly higher than that of other three groups on the 4^{th}, 7^{th} and 35^{th} day ($P<0.05$), and there was no difference between Group 4 and other groups on the 49^{th} and 120^{th} day ($P>0.05$).

Figure 7 The density of EGFP positive cells in tissue stretched preparation on the 35^{th} day. The number of transfected cells in Group 4 (B) was more than that in Group 1 (A). EGFP expression mainly showed in RPE cells (C).

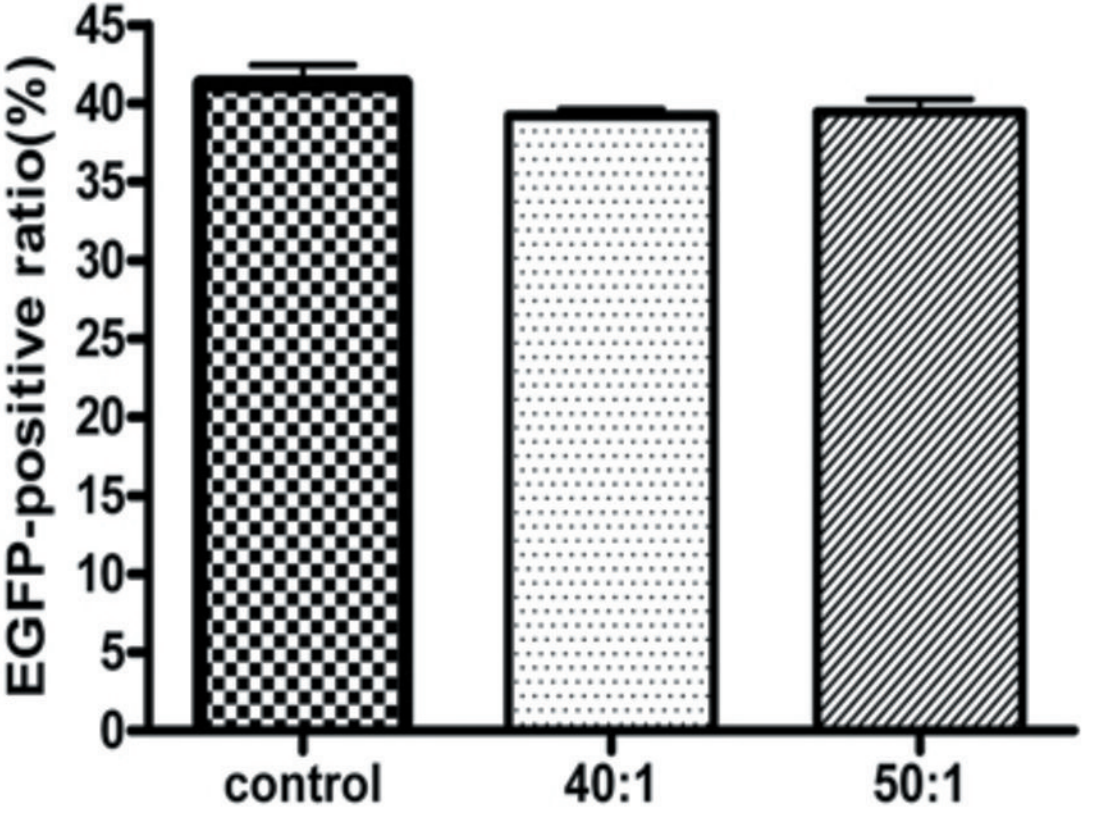
Figure 8 Histology showed no evident tissue damage after UTMD mediated AAV transfection.

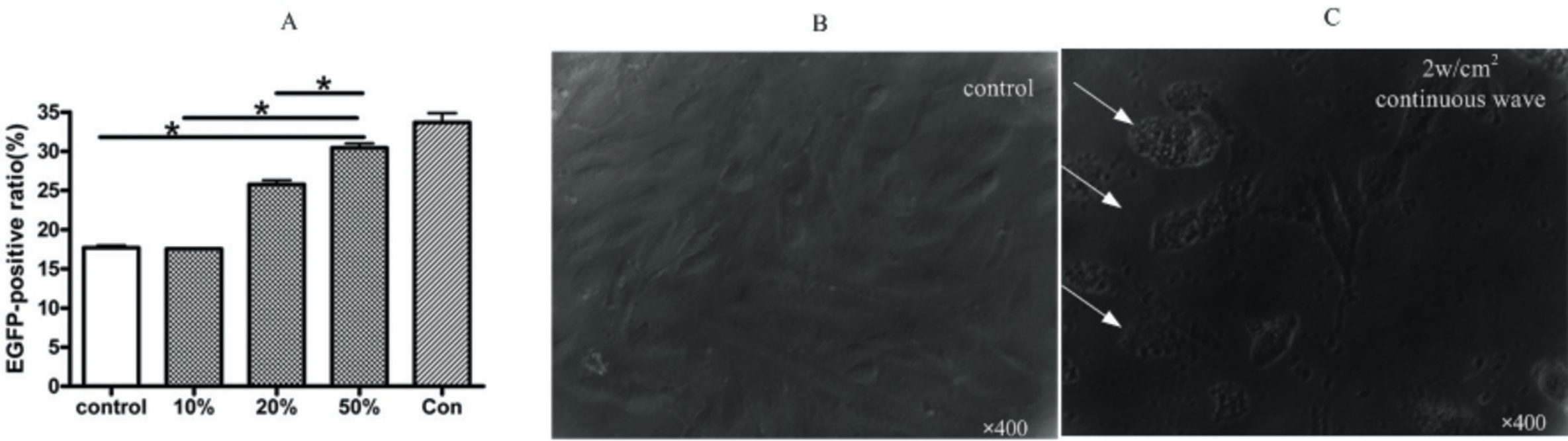
Figure 9 Frozen sections of fundus oculi showed EGFP expression major in the layer of retina.

A

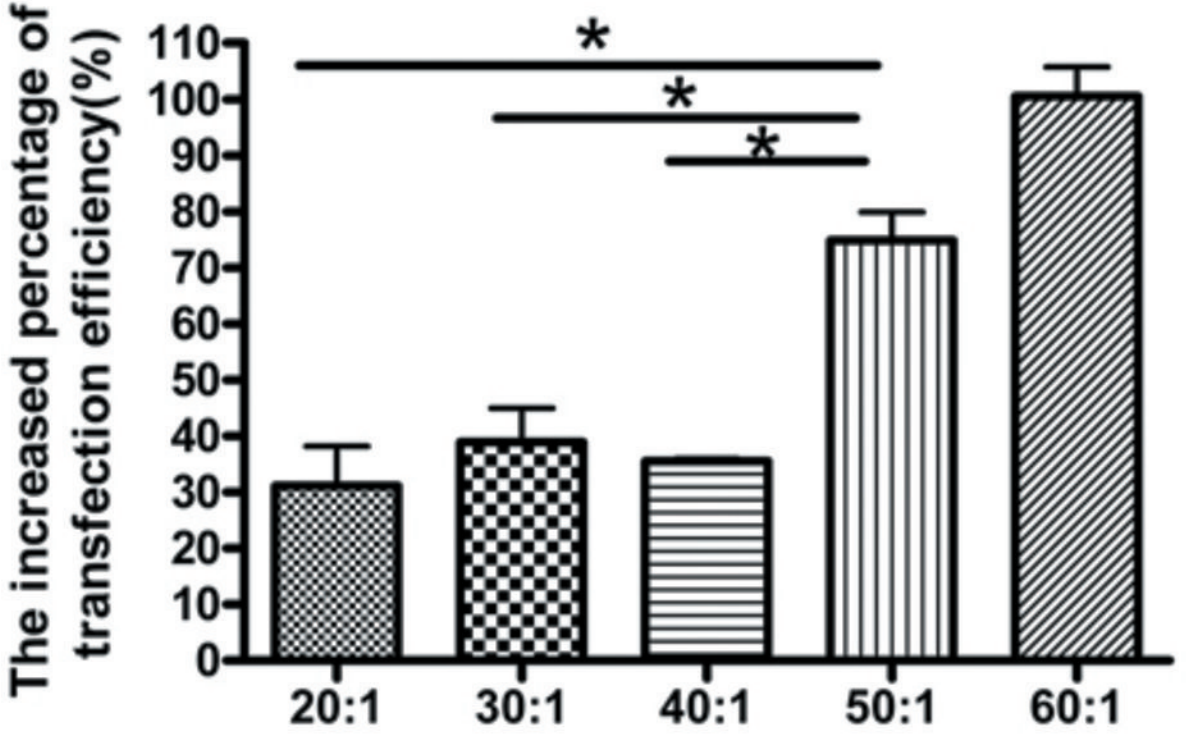

EGFP-positive ratio(%)
25
20
15
10
5
0
control
1w/cm2,60s
2w/cm2,60s
3w/cm2,60s
1w/cm2,120s
2w/cm2,120s
3w/cm2,120s

B

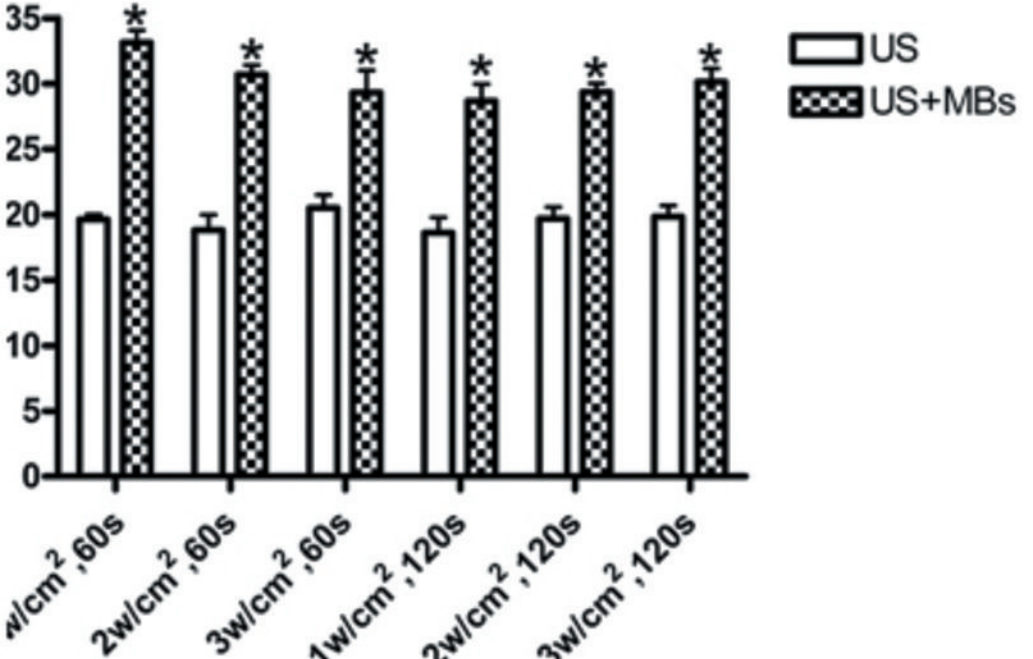

EGFP-positive ratio(%)
45
40
35
30
25
20
15
10
5
0
control
40:1
50:1

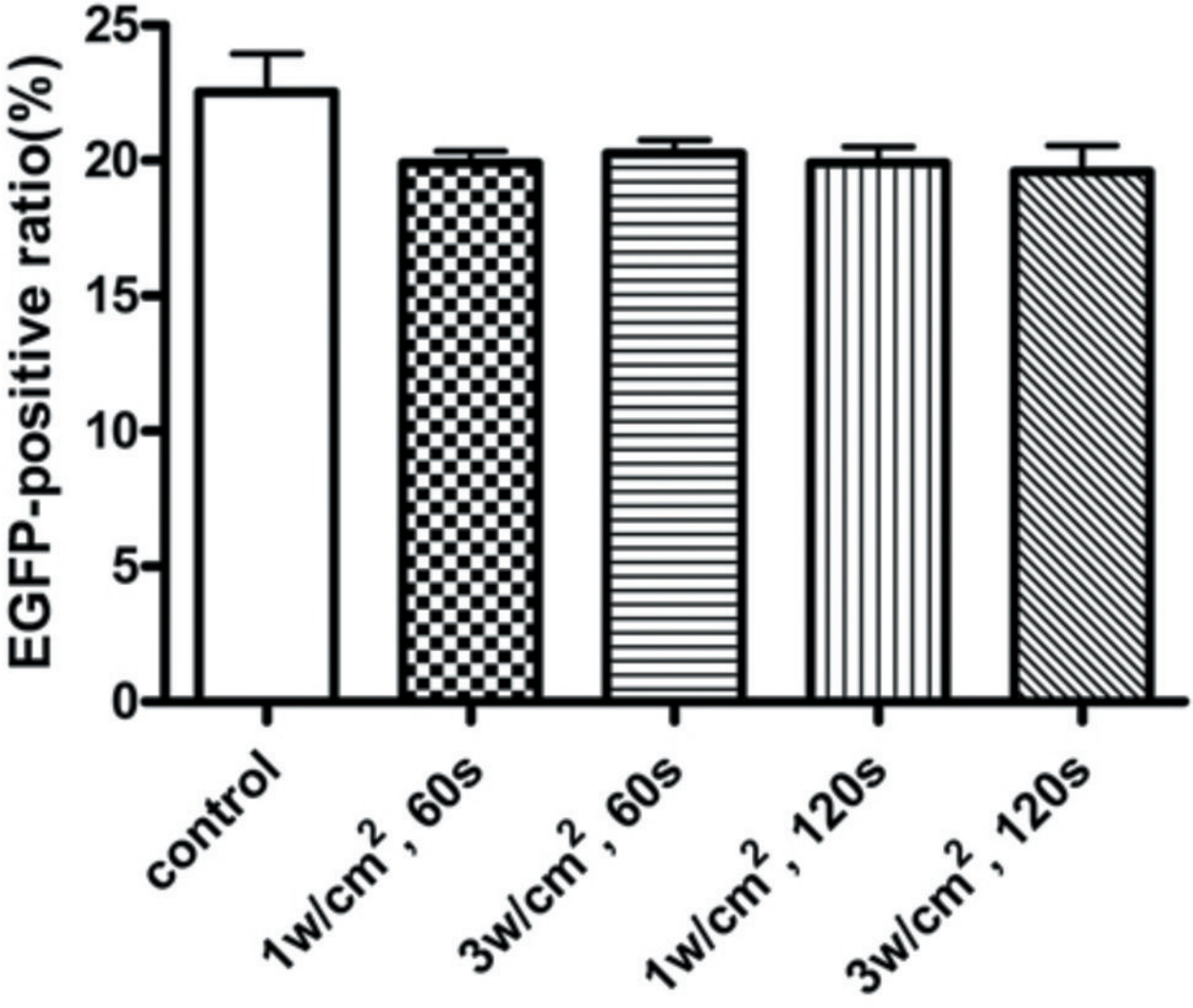

A
B
C
EGFP-positive ratio(%)
35
30
25
20
15
10
5
0
control
10%
20%
50%
Con
*
*
*
control
×400
2w/cm²
continuous wave
×400

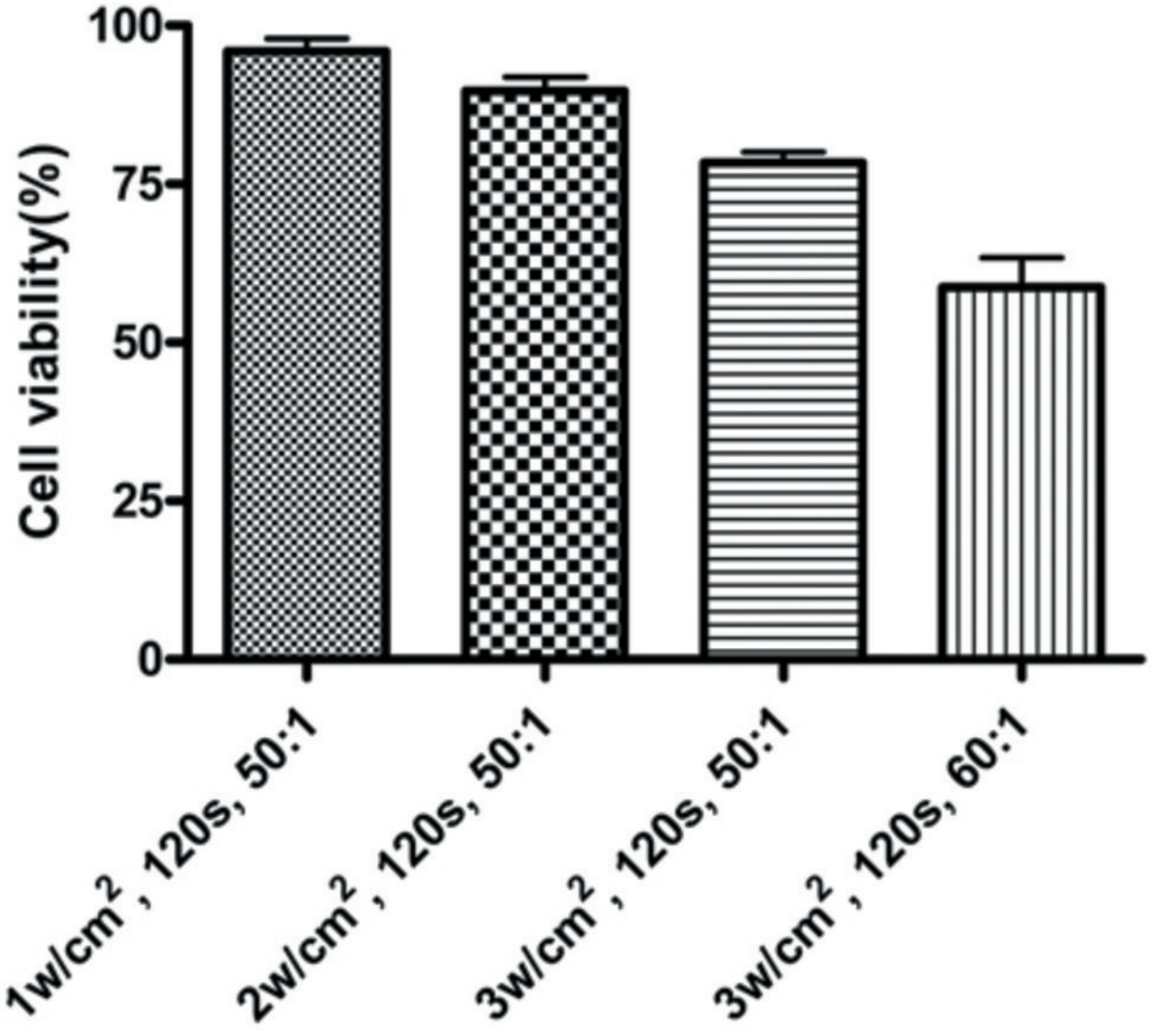
A

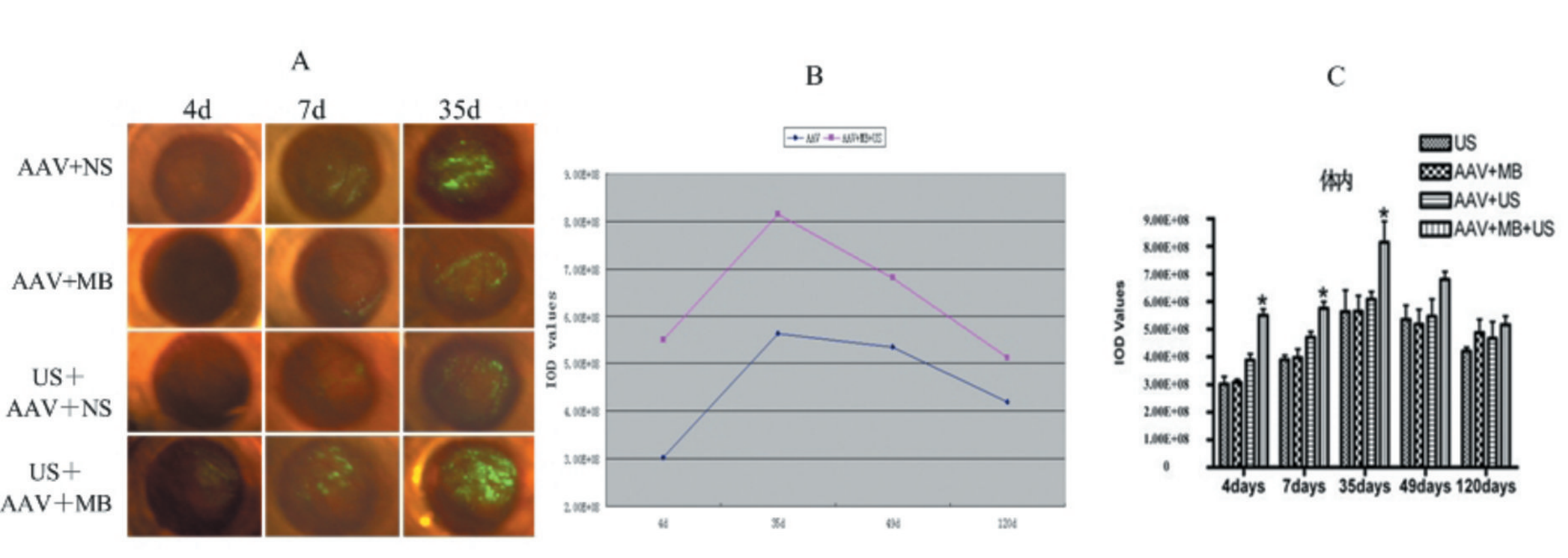

The increased percentage of transfection efficiency(%)
110
100
90
80
70
60
50
40
30
20
10
0
*
*
*
20:1
30:1
40:1
50:1
60:1

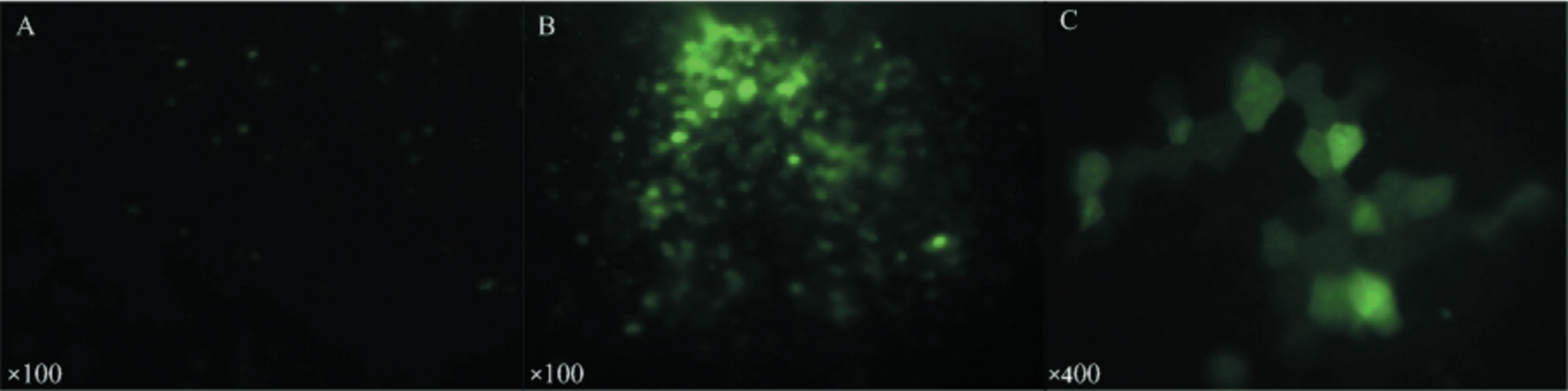
B

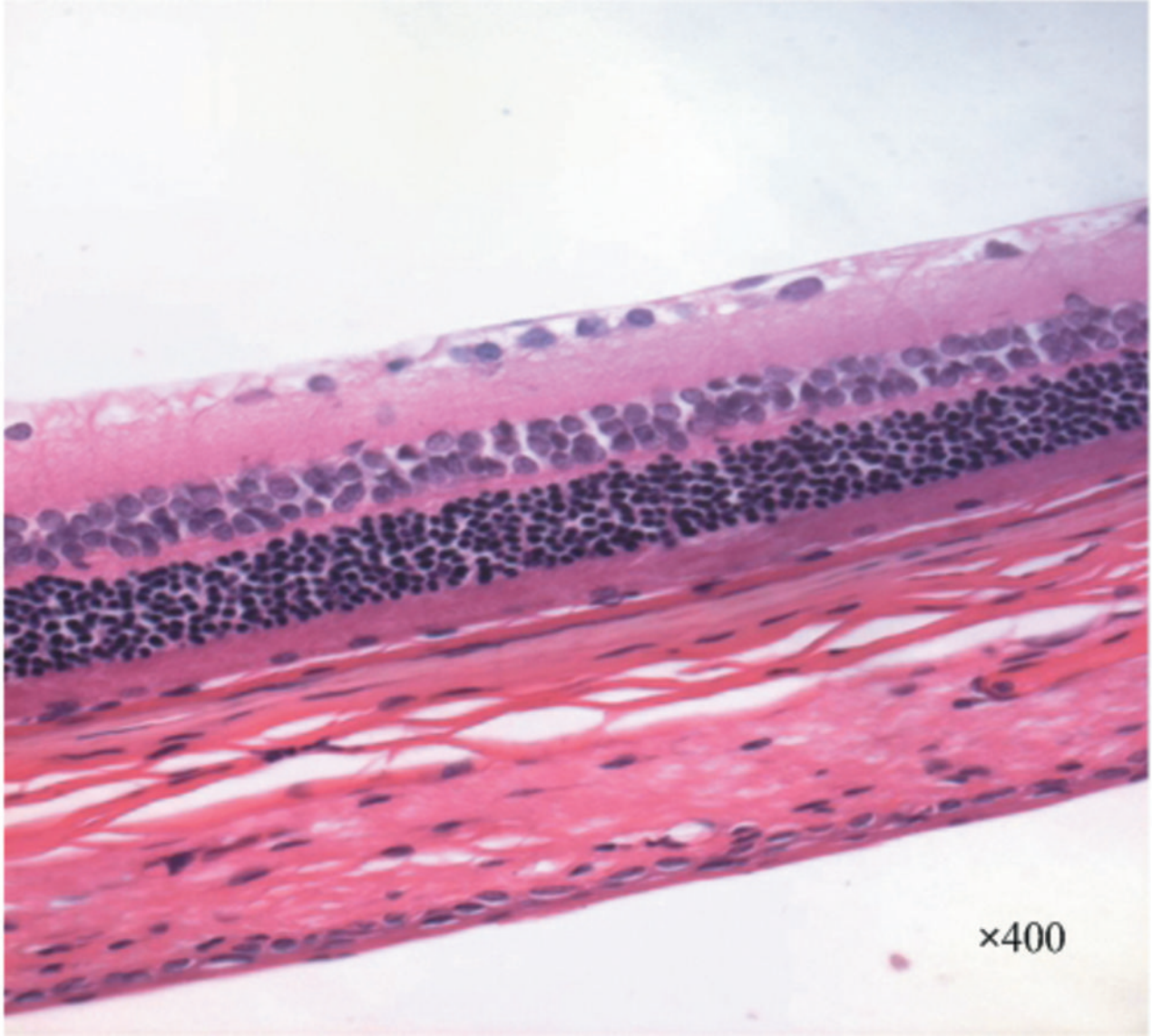

35
30
25
20
15
10
5
0
US
US+MBs
*
w/cm^2,60s
2w/cm^2,60s
3w/cm^2,60s
1w/cm^2,120s
2w/cm^2,120s
3w/cm^2,120s

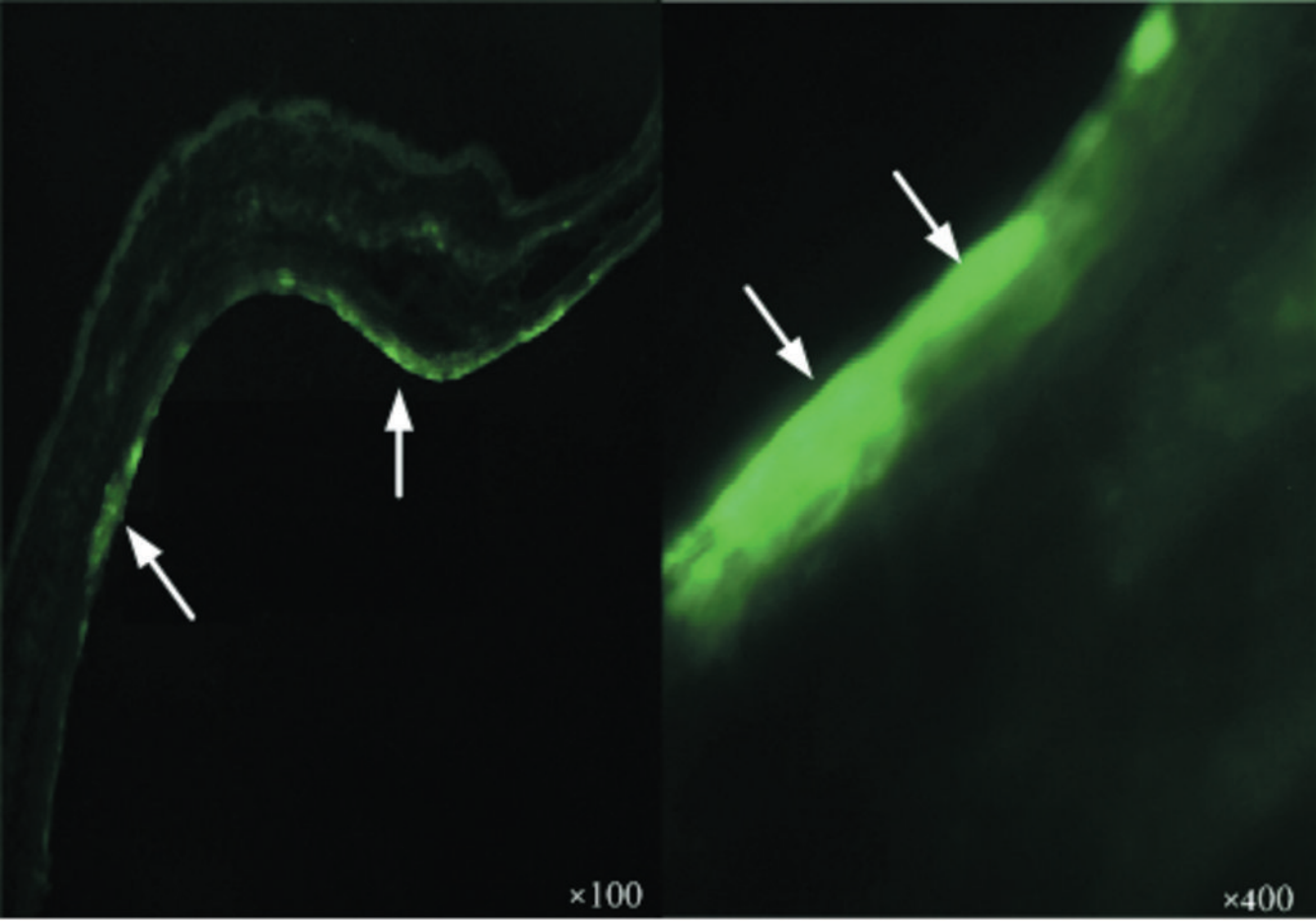

EGFP-positive ratio(%)
25
20
15
10
5
0
control
1w/cm², 60s
3w/cm², 60s
1w/cm², 120s
3w/cm², 120s


100
75
50
25
0
Cell viability(%)
1w/cm^2, 120s, 50:1
2w/cm^2, 120s, 50:1
3w/cm^2, 120s, 50:1
3w/cm^2, 120s, 60:1


A
4d
7d
35d
AAV+NS
AAV+MB
US+
AAV+NS
US+
AAV+MB
B
IOD values
C
US
AAV+MB
AAV+US
AAV+MB+US
IOD Values
4days
7days
35days
49days
120days


A
×100
B
×100
C
×400


×400


×100
×400