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Original Article

Ultrasound-microbubble enhances bioavailability of neuro growth factor in neuro-retina after intravitreal injection in rabbits

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Abstract: This study was to determine and compare the distribution and concentration of mNGF between two groups with and without ultrasound microbubble in rabbit's eyes after intravitreal injection. Intravitreal injection of mNGF (18 μg/100 μL) with and without mixing of microbubbles were performed on 48 New Zealand rabbits (96 eyes). The left eyes (48 eyes, group A) were intravitreally injected with 18 μg/100 μL of mNGF only at supertemporal sclera apart from 3 mm cornea sclera edge. The right eyes (48 eyes, group B) were intravitreally injected with SonoVue (100 μL), a type of microbubble, followed by 100 μL of mNGF at the same time. After the injections, the right eyes (group B) were immediately radiated with ultrasound of 1 MHZ frequency, 0.5 W/cm^2 ultrasonic intensity for 60 s. Then, the rabbits were sacrificed at points of 0.5, 1, 2, 3, 4, 6, 12 and 24 hours after injection, with 6 rabbits at each time point. The eye tissue was collected for determination of mNGF concentration in dissected ocular tissue of vitreous body, retina and optic nerve by high performance liquid chromatography (HPLC). After the injection of same amount of mNGF with or without SonoVue and ultrasound, the concentration of mNGF in vitreous decreased lineally with the time elapsed. The kinetics followed the pattern of first-order. In group A, the concentration of mNGF changed from 2.186±0.089 ng/mg to 0.061±0.001 ng/mg without SonoVue and ultrasound, and in group B, the concentration of mNGF changed from 1.949±0.048 ng/mg to 0.058±0.002 ng/mg with SonoVue and ultrasound. Concerning the concentration of mNGF, there is significant difference between two groups at 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 24 h after injection (P=0.000, 0.001, 0.000, 0.021, 0.047, 0.008 and 0.012, respectively). The injection of mNGF with SonoVue and ultrasound resulted in a quicker pervasion and a lower concentration in vitreous than the injection of mNGF only at all the time points. The distribution of mNGF in retina and optic nerve after the injection with or without SonoVue followed a two-phase pattern. Without SonoVue and ultrasound in group A, the mean values of concentration of mNGF were 0.152±0.010 ng/mg, 0.193±0.008 ng/mg, 0.257±0.011 ng/mg, 0.385±0.013 ng/mg, 0.277±0.014 ng/mg, 0.180±0.007 ng/mg, 0.064±0.010 ng/mg and 0.002±0.000 ng/mg at 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 12 h and 24 h, respectively in retina; and they were 0.080±0.003 ng/mg, 0.110±0.009 ng/mg, 0.148±0.007 ng/mg, 0.222±0.012 ng/mg, 0.246±0.010 ng/mg, 0.122±0.004 ng/mg, 0.029±0.008 ng/mg and 0.000±0.000 ng/mg at 0.5 h, 1 h, 2 h, 3h, 4 h, 6 h, 12 h and 24 h, respectively in optic nerve. With SonoVue and ultrasound in group B, all of the mean value (except at 24 h due to its value of 0.000±0.000 ng/mg in optic nerve) was higher than that in group A, which was 0.194±0.012 ng/mg (P=0.004), 0.228±0.007 ng/mg (P=0.000), 0.316±0.012 ng/mg (P=0.000), 0.442±0.011 ng/mg (P=0.002), 0.306±0.008 ng/mg (P=0.000), 0.193±0.005 ng/mg (P=0.000), 0.083±0.004 ng/mg (P=0.000) and 0.003±0.000 ng/mg (P=0.000) at 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 12 h and 24 h, respectively in retina; and they were 0.101±0.006 ng/mg (p=0.000), 0.141±0.006 ng/mg (P=0.000), 0.189±0.014 ng/mg (P=0.002), 0.257±0.004 ng/mg (P=0.001), 0.301±0.012 ng/mg (P=0.001), 0.140±0.005 ng/mg (P=0.001), 0.042±0.007 ng/mg (P=0.001) and 0.000±0.000 ng/mg at 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 12 h and 24 h, respectively in optic nerve. The differences in all time point between group A and B in retina were statistical significance, while in all time point except for the time point of 24 h in optic nerve were also statistical significance (P < 0.05). The content reached a peak in 3 hour in retina (0.385±0.013 ng/mg in group A, 0.442±0.011 ng/mg in group B) and in 4 hour in optical nerve (0.246±0.010 ng/mg in group A, 0.301±0.012 ng/mg in group B) after the injection in both groups. At the time point of 12 hour, there was a 1.29 fold higher content in retina in the group with microbubble than that without the microbubble, and there was a 1.44 fold higher content in the optic nerve in the group with microbubble than that without the microbubble. The higher concentration of the mNGF at whole time course in both retina and optic nerve was always associated with the injection of the combined mNGF and SonoVue, mediated by ultrasound. The allocation of the mNGF maintained a longer and higher existing

of the agent in retina than that in optic nerve. Intravitreal injection of mNGF together with ultrasound microbubble delivery can generated a higher concentration in the target tissue of retina and optic nerve than the injection with mNGF only. Ultrasound-microbubble enhances bioavailability of mNGF in neuro-retina after intravitreal injection in rabbits.

Keywords: Ultrasound microbubble, mouse nerve growth factor, intravitreal injection, distribution

Introduction

Nerve growth factor (NGF) is one of the neurotropic factors and plays an important role in supporting central and peripheral neurons for their growth, development, differentiation, regeneration [1-4]. NGF contains the activated substances and bioactive factors that can increase protein synthesis of nerve cell, supply energy to cell and regulate the metabolism of nucleic acid and sugar, thus promote the regeneration of the nerve cells [5-7]. When nerve cells was damaged, exogenous NGF could reduce the degree of damage to neuronand promote the regeneration of nerve fibers and the recovery of neural function [3-5].

Currently, intramuscular injection type of NGF extracted from mouse submandibular gland has been accepted in clinical application for various retinal diseases including glaucoma [6], diabetic retinopathy [7], retinitis pigmentosa [8] and optic nerve contusion [9]. NGF can alleviate nerve damage, inhibit apoptosis and promote the repair of damaged nerve. It was reported that NGF could increase the survival rate of RGCs by 30% compared with the control group after optic nerve injury [10].

Due to blood-eye barrier and the anatomy of the eye, the local effective concentration of NFG in retina and optic nerve is very low with intramuscular injection [11-14]. Since local administration such as eye drop and intravitreal injection has clearer target, it has been the common treatment for eye disease. Colafrancesco V [15] reported that NGF eye drop administration exerts a protective effect on animal models of retinal degeneration in glaucoma and diabetic retinopathy. But it is also reported that a drop of drug to the posterior segment of eye is less than 5% of concentration [11-14]. While in intravitreal injection, the drug directly releases to the vitreous cavity, retina and optic nerve, so as to obtain higher drug concentration. Moreover the blood-retinal barrier can keep drug in eye for longer time. Sivilia S [16] reported that a single intravitreal NGF injection protects retina and optic nerve from degeneration due to vascular injury. This effect is also mediated by an increased synthesis of endogenous NGF due to the mechanical lesion associated with intraocular delivery.

Since the blood-eye barrier of eye could be a limitation of optimal concentration of NFG in retina and optic nerve after intramuscular injection. The recent development of ultrasound contrast agent microbubbles represents a new method for topical treatment of eye diseases. These microbubbles are blasted using specific ultrasound energy and the drugs can be directly released at the target tissues as a targeted therapy. Direct injection of therapy agents coupled with release devices to vitreous cavity may be an ideal approach to facilitate the distribution of compounds to retina and optic nerve. Ultrasound microbubble composed by the filling gas and shell of phospholipid can produce cavitation and acoustic hole after ultrasonic irradiation [17]. The effect of sound hole produces different quantity and sizes of holes in the cell membrane and increases local capillaries and permeability, so as to make drug easier entry into cells [18-22].

In order to test whether intravitreal injection with mNGF combined with ultrasound microbubble can increase the bioavailability of mNGF in ocular tissue, we compared the distribution and concentration of mNGF between two treatments with and without ultrasound microbubble in rabbit's eyes after intravitreal injection of mNGF.

Materials and methods

Reagent and preparation

Mouse Nerve Growth Factors (mNGF) were purchased from Xiamen Beida Biological Engineering Company (Beida, China). A bottle of dried powder of mNGF (18 μg) was mixed with 0.1 mL of 0.9% sodium chloride (18 μg/100 μL) before use. Microbubble (SonoVue, sulfur hexafluoride, 2-4 μm in diameter) was purchased from

Bracco (Italy). Freeze-dried powder (59 mg) SonoVue were mixed with 5 mL of 0.9% sodium chloride and shaken to generate microbubble before injection and used within 6 hours.

Animals and treatment

The animal studies were conducted in compliance with the ARVO statement for the use of animals, and all animal experiments were performed under protocols approved by the Institutional Animal Care of Shenzhen Eye Hospital. Forty eight healthy NewZealand's rabbits (1.5 to 1.7 kg in body weight) were purchased from Medical Experimental Animal Center of Guangdong Province and maintained in Animal Center of the affiliated Shenzhen hospital of Peking University. The rabbits had clear cornea, transparent lens, clear vitreous and fundus examined with slit lamp and direct ophthalmoscope. Sumianxin (0.2 mL/kg) was intramuscularly injected for anesthesia before intravitreal injection. The left eyes (48 eyes, group A) were intravitreally injected with 18 μg/100 μL of mNGF at supertemporal sclera apart from 3 mm corneasclera edge. The right eyes (48 eyes, group B) were intravitreally injected with SonoVue (100 μL), followed by 100 μL of mNGF at same position. After the injection, the right eyes were immediately radiated with ultrasound at 1 MHZ frequency, 0.5 W/cm^2 in intensity for 60 seconds with ultrasound therapeutic gene transfection apparatus [23] (Chongqing Medical University, Institute of Ultrasound Imaging).

After that, rabbits were respectively sacrificed with 6 rabbits together at each time point of 0.5, 1, 2, 3, 4, 6, 12 and 24 hour after injection. The eyeballs with optic nerve were excavated, then the vitreous was extracted with 1 mL syringe and the rest of the ocular tissue was briefly fixed in ethanol (99.5%). All of the retina and optic nerves with discus optics were dissected, weighed and recorded respectively.

The determination of mNGF with high performance liquid chromatography (HPLC)

Vitreous body were centrifuged for 30 min at 15000 RPM and the supernatants were collected for HPLC analysis. The retina and optic nerve were cut into pieces with micro retina scissor and homogenized in 1 mL anhydrous ethanol on ice and homogenate were centrifuged for 30 min at 15000 RPM and the supernatants were collected for HPLC analysis.

mNGF was separated by HPLC (Agilent 1260, Wilmington, DE, USA) using Zorbax SB_C18 column (150 mm×4.6 mm, 5 μm) and detected by diode array detector at the maximum absorption wavelength of 220 nm. The HPLC condition was set as the injection volume of 100 μL, mobile phase of 20% acetonitrile in 0.1% trifluoroacetic acid at flow rate of 1.0 mL/min. The retention time of mNGF is about 13 min under above conditions. The area of mNGF peak was compared with the standard curve and results were present as ng mNGF per mg tissue weigh (ng/mg).

Statistical analysis

The comparison of the means of mNGF contents in each tissue at each time point was conducted by paired t-test using SPSS13.0 software. The value of $P < 0.05$ was considered as statistical significance.

Results

After the injection of 18 μg/100 μL mNGF in group A, and 18 μg/100 μLl mNGF plus 100 μL SonoVue (mediated by ultrasound) in group B, the concentration of mNGF in vitreous decreased lineally with the time elapsed. In group A, the concentration of mNGF changed from 2.186±0.089 ng/mg to 0.061±0.001 ng/mg without SonoVue and ultrasound, and in group B, the concentration of mNGF changed from 1.949±0.048 ng/mg to 0.058±0.002 ng/mg with SonoVue and ultrasound. The kinetics followed the pattern of first-order. It took approximately 3 hours for the concentration to decrease to one-half its initial concentration in both groups, that is, in group A, from 2.186±0.089 ng/mg to 1.072±0.048 ng/mg, as well as in group B, from 1.949±0.048 ng/mg to 0.976±0.060 ng/mg. Concerning the concentration of mNGF, there were significant differences between two groups at 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h and 24 h after injection (P=0.000, 0.001, 0.000, 0.021, 0.047, 0.008 and 0.012, respectively), except the time point of 12 h (P = 0.161). The injection of mNGF with SonoVue and ultrasound resulted in a quicker pervasion and a lower concentration in vitreous body than the injection of mNGF only at all the time points,

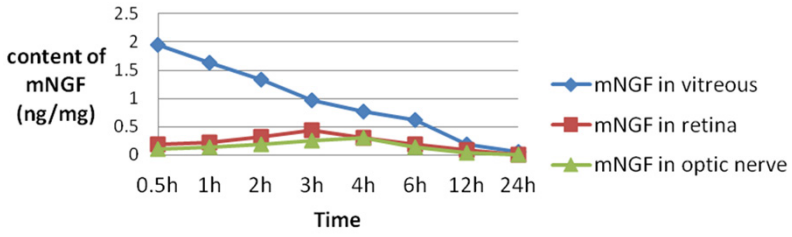

Figure 1. The content of mNGF in each eye tissue in group mNGF with SonoVue. After intravitreal injection of mNGF with SonoVue, the concentration of the protein in vitreous decreased lineally with the time elapsed. The kinetics followed the pattern of first-order. The distribution of mNGF in retina and optic nerve after intravitreal injection with SonoVue followed a two-phase pattern.

suggesting a redistribution of the mNGF in the presence of microbubble (**Figure 1**; **Table 1**).

The distribution of mNGF in retina and optic nerve after the injection with or without SonoVue followed a two-phase pattern (**Figure 1**; **Tables 2** and **3**). Without SonoVue and ultrasound in group A, the mean values of concentration of mNGF were 0.152±0.010 ng/mg, 0.193±0.008 ng/mg, 0.257±0.011 ng/mg, 0.385±0.013 ng/mg, 0.277±0.014 ng/mg, 0.180±0.007 ng/mg, 0.064±0.010 ng/mg and 0.002±0.000 ng/mg at 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 12 h and 24 h respectively in retina; they were 0.080±0.003 ng/mg, 0.110±0.009 ng/mg, 0.148±0.007 ng/mg, 0.222±0.012 ng/mg, 0.246±0.010 ng/mg, 0.122±0.004 ng/mg, 0.029±0.008 ng/mg and 0.000±0.000 ng/mg at 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 12 h and 24 h respectively in optic nerve. With SonoVue and ultrasound in group B, all of the mean values (except this time point of 24 h due to its value of 0.000±0.000 ng/mg in optic nerve) were higher than that in group A, which was 0.194±0.012 ng/mg (P=0.004), 0.228±0.007 ng/mg (P=0.000), 0.316±0.012 ng/mg (P=0.000), 0.442±0.011 ng/mg (P=0.002), 0.306±0.008 ng/mg (P=0.000), 0.193±0.005 ng/mg (P=0.000), 0.083±0.004 ng/mg (P=0.000) and 0.003±0.000 ng/mg (p=0.000) at 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 12 h and 24 h respectively in retina; they were 0.101±0.006 ng/mg (p=0.000), 0.141±0.006 ng/mg (P=0.000), 0.189±0.014 ng/mg (P=0.002), 0.257±0.004 ng/mg (P=0.001), 0.301±0.012 ng/mg (P=0.001), 0.140±0.005 ng/mg (P=0.001), 0.042±0.007 ng/mg (P=0.001) and 0.000±0.000 ng/mg at 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 12 h and 24 h respectively in optic nerve (**Tables 2** and **3**). The differences in all time points between group A and B in retina were statistically significant, while in all time points except for the time point of 24 h in optic nerve were also statistically significant ($P < 0.05$).

The content reached a peak in 3 hour in retina (0.385±0.013 ng/mg in group A and 0.442±0.011 ng/mg in group B) and in 4 hour in optical nerve (0.246±0.010 ng/mg in group A and 0.301±0.012 ng/mg in group B) after the injection in both groups. At the time point of 12 hour, there was a 1.29 fold higher content in retina in the group with microbubble than that without the microbubble (**Table 2**), and there was a 1.44 fold higher content in the optic nerve in the group with microbubble than that without the microbubble (**Table 3**). The higher concentration of the mNGF at whole time course in both retina and optical nerve was always associated with the injection of the combined mNGF and SonoVue. The allocation of the mNGF maintained a longer and higher existing of the agent in optic nerve than that in retina.

Discussion

In this study, the intravitreous administration of mNGF quickly dispersed the protein through posterior segment. The dwell time of mNGF in the target tissue of retina and optic nerve is well maintained due to probably the blood-retina barrier formed by the retinal pigment epithelium (RPE) and the closely connected retinal capillary that can prevent the macromolecular substance spread out of the eye.

Vitreous body is composed of 98% water and 2% collagen, so water soluble drugs such as mNGF after intravitreal injection is easy to diffuse from the vitreous to other tissues in the eye. Dispersion and discharge pathway of intravitreous drus mainly includes anterior segment pathway and posterior segment pathway [24, 25]. The former refers to the canal from vitreous to the lens, posterior chamber, the iris and ciliary body into the anterior chamber, and then through the Schlemm tube of trabecular meshwork, expels with aqueous humor, partially disperse to cornea and conjunctive. The latter refers to the canal from vitreous to the retina

Table 1. The content of mNGF (ng/mg) in vitreous at each time point (ng/mg)

Group	Time points (hours)							
	0.5	1	2	3	4	6	12	24
A-mNGF	2.186±0.089	1.883±0.043	1.523±0.063	1.072±0.048	0.839±0.034	0.654±0.020	0.202±0.004	0.061±0.001
B-mNGF+SonoVue	1.949±0.048*	1.636±0.062*	1.337±0.085*	0.976±0.060*	0.778±0.029*	0.618±0.011	0.196±0.007*	0.058±0.002
Fold change (%)	89	86	87	91	92	94	97	95
P value	0.000	0.001	0.000	0.021	0.047	0.008	0.161	0.012

The contents of mNGF of each time point in vitreous of the group mNGF were higher than group mNGF+SonoVue. The data is the average from the measurement of 6 eyes.

Table 2. The content of mNGF (ng/mg) in retina at each time point

Group	Time points (hour)							
	0.5	1	2	3	4	6	12	24
A-mNGF	0.152±0.010	0.193±0.008	0.257±0.011	0.385±0.013	0.277±0.014	0.180±0.007	0.064±0.010	0.002±0.000
B-mNGF+SonoVue	0.194±0.012*	0.228±0.007*	0.316±0.012*	0.442±0.011*	0.306±0.008*	0.193±0.005*	0.083±0.004*	0.003±0.000*
Fold change (%)	127	118	122	114	110	107	129	150
P value	0.004	0.000	0.000	0.002	0.006	0.000	0.014	0.000

The contents of mNGF of each time point in retina of the group mNGF+SonoVue were higher than group mNGF. The data is the average from the measurement of 6 eyes.

Table 3. The content of mNGF (ng/mg) in optic nerve at each time point

Group	Time points (hour)							
	0.5	1	2	3	4	6	12	24
A-mNGF	0.080±0.003	0.110±0.009	0.148±0.007	0.222±0.012	0.246±0.010	0.122±0.004	0.029±0.008	0.000±0.000
B-mNGF+SonoVue	0.101±0.006*	0.141±0.006*	0.189±0.014*	0.257±0.004*	0.301±0.012*	0.140±0.005*	0.042±0.007*	0.000±0.000
Fold change (%)	126	128	127	115	122	114	144	-
P value	0.000	0.000	0.002	0.001	0.001	0.001	0.001	-

The contents of mNGF of each time point in optic nerve of the group mNGF+SonoVue were higher than group mNGF. The data is the average from the measurement of 6 eyes.

and choroid, diffuse to the sclera, especially choroid capillary, as the main pathway of drugs. According to the results of our study, mNGF in vitreous mainly disperse and discharge through posterior segment pathway. The retina is closely connected to vitreous, thus mNGF can directly disperse to retina through vitreous body. In addition, blood-retina barrier formed by the retinal pigment epithelium (RPE), closely connected to retinal capillary wall, can prevent macromolecular substances and water-soluble drugs spread out of eye, meanwhile prolong the dwell time of drugs.

Our results also indicated that microbubble ultrasound mediated intravitreal injection substantially enhanced the distribution of mNGF in the target tissue of retina and optic nerve. It is because that by ultrasound microbubble intravitreal delivery, mNFG diffused faster from vitreous to retina and optic nerve, then greater content access to these tissues. Due to targeting function of ultrasound microbubble, drug in local tissue can achieve high content and thus provide a new mode of drug delivery for eye diseases, especially the retinal diseases. This technology has been tested in improving the pigment epithelium-derived factor gene transfection and can effectively restrain the development of the choroid new blood vessels [18]. The number of RGCs significantly increased in Memantine injection together with ultrasound microbubble [19]. Ultrasound microbubble mediated targeting drug delivery has the advantage of low immunogenicity and low toxicity. This technology has been applied in drug delivery, gene therapy, thrombolysis and tumor treatment and other fields [17, 18, 20, 22].

The ultrasound microbubble used in current study has a diameter between 1 to 8 microns. It is a micro bubble with sulfur hexafluoride sheathing by a new type of outer lipid membrane, with average diameter of 2.5 microns and 90% diameter less than 8 microns [21]. There are three kinds of mechanism that ultrasound interact with biological organization: mechanical effect, thermal effect and cavitation effect [20]. Cavitation effect refers to that when sound waves go through liquid, tiny air bubbles in the liquid (cavitation nuclei) vibrate periodically with change of sound pressure. It rapidly expanse in half phase with negative pressure, while rapidly contract in half phase with positive pressure, leading to implosion [26]. Due to the cavitation effect and target effect, microbubble technique is applied from the original field of imaging diagnosis into therapeutic domain. Compared with the traditional gene targeting carrier, the technology is considered to be a new kind of noninvasive drug delivery system with advantage as low immunogenicity, low toxicity, organ tissue specificity and repeatability [22]. Therefore, it is of great significance to introduce gene or drug therapy for eye diseases [27-30].

Ultrasound microbubble contrast agent can not only increase the effective concentration in target organ but also maintain the original structure of drugs, thus provides a new model for targeted therapy in various diseases. In this study, there was no difference in drug distribution time between with and without ultrasound microbubble, suggesting that ultrasound microbubble does not affect the metabolic properties of mNGF itself.

In conclusion, intravitreal injection of mNGF together with ultrasound microbubble delivery can generate a higher concentration in the target tissue of retina and optic nerve than the injection with mNGF only. This novel approach is warranted for further study in the future.

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Disclosure of conflict of interest

None.

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