Enhancement of naked FIX minigene expression by chloroquine in mice

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KEY WORDS chloroquine; factor IX; naked plasmids

ABSTRACT

AIM: To study the effect of chloroquine on the expression of human clotting factor IX (hFIX) in mice. **METHODS:** Hydrodynamics-based naked DNA plasmid administration was performed by tail vein injection of 10 μg of pCMV-hFIX and chloroquine (0, 100, 200, and 500 μmol/L) in 2.2 mL of Ringer' solution within 6-7 s, the level and stability of hFIX expression, liver damage and toxicity were then examined. **RESULTS:** The maximum expression of hFIX level was 4.4±1.8 mg/L at 8 h after injection, 9.7±1.6 mg/L at 24 h only existed in 200 μmol/L chloroquine-treated animals, which is 3-4 fold higher than that of control (P<0.01). There is no significant difference observed among all the treated groups, 3 d later. Transaminase level and liver histological study showed the damage of liver was not related to chloroquine (P>0.05). **CONCLUSION:** Chloroquine can enhance and sustain exogenous gene expression *in vivo* without side effect under our experimental conditions.

INTRODUCTION

Development of nonviral naked DNA transfection is attractive since they have several advantages over viral-based vectors, eg, simplicity of construction, ease of large-scale production, cost effectiveness, less toxic, nonimmunogenic and the introduced exogenous genes do not integrate into the host genome. However, the relatively low and transient nature of gene expression limited the application of naked DNA transfer system.

Liu[1] *et al* and Zhang[2] *et al* have developed a technique for expressing exogenous genes in mice by rapid tail vein injection of a large volume of naked DNA solution, the "hydrodynamics-based procedure". Liu achieved 45 mg of luciferase protein per gram of liver by a single tail vein injection of 5 mg of plasmid DNA into mouse. Histochemical analysis using β-galactosidase gene revealed that approximately 40 % of hepatocytes expressed the transgene. However, this method of delivery resulted in a rapid decline in transgene expression. The short duration of transgene expression remained a major obstacle for the implementation of nonviral DNA vectors in further studies. This is probably due to a gradually loss of the episomal plasmid and/or the silencing of the template[3].

Chloroquine is a medical drug of a 4-aminoquinoline structure firstly synthesized and studied by Resochin in Germany in 1934, and was used widely for both treatment and prophylaxis of malaria[4]. Since 1964, chloroquine was utilized in the chemoprophylaxis of malaria and later became well known in the successful treatment of extraintestinal amebiasis, discoid lupus erythematosus, insulin-independent diabetes and rheumatoid arthritis[5] and most recently for treatment of Alzheimer

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disease[6] and AIDS[7].

Chloroquine is also known to enhance transfer efficiency *in vitro*[8,9]. Possible reason is that chloroquine interfere with the endocytosis process, in particular, raise the luminal pH of endocytotic vesicles and reduce the ligand delivery to lysosomes and the intracellular degradation by lysosomal enzymes. In addition, chloroquine can form complexes with DNA and might protect DNA molecules from nuclease degradation[10].

Chloroquine has been frequently used for gene transfection studies in cell culture[11], but has not been reported to be successful for enabling gene transfection *in vivo*. Recently, Zhang[12] firstly reported chloroquine promoted non-viral gene delivery to rat liver via portal vein and bile duct. Intraperitoneal injection and oral chloroquine resulted in increasing levels of luciferase reporter gene expression, following polylysinemolossion/DNA complexes delivery via bile duct in rats. In this study, we systematically investigated the effect of chloroquine on exogenous gene expression in mice by hydrodynamics-based naked plasmid administration.

MATERIALS AND METHODS

Plasmid preparations The CMVβ plasmid containing the β-galactosidase gene under the control of the CMV promoter (Clontech, Palo Alto, CA, USA) and the CMV-FIX plasmid was constructed in our lab. Human FIX minigene was driven by CMV promoter. There was a 800-bp intron I between exon 1 and exon 2. Plasmid DNA was purified by the method of CsCl-ethidium bromide gradient centrifugation. The purified DNA was diluted to 1 g/L and frozen at -20 ºC.

Cytotoxicity of chloroquine Chloroquine (Sigma Co) was prepared at sterile pure water to 100 mmol/L and stored at -20 ºC. Mice was given chloroquine at different concentration (0, 100, 200, 500, and 1000 μmol/L) in 2.2 mL of Ringer's solution (147 mmol/L NaCl, 4 mmol/L KCl, 1.13 mmol/L $CaCl_2$) by the tail vein injections within 6-7 s. Twenty-four h later, mice were killed and the liver sections were stained by a hematoxylin/eosin staining.

Animal experiments Six-week-old male ICR mice were purchased from BK Inc (Shanghai) and housed under SPF conditions. In brief, the tail vein injections were performed by injecting through a 27-gauge needle 10 μg of pCMV- hFIX and chloroquin (0, 100, 200, and 500 μmol/L) in 2.2 mL of Ringer's solution within 6-7 s. Scheduled blood samples were collected from the retro-orbital plexus.

Localization of β-galactosidase gene expression We harvested the lung, heart, liver, spleen, and kidney for X-gal staining 1 d after the injection of 10 μg pCMVβ and 200 μm chloroquine, embedded in Tissue-Tek OCT compound (Sakura Finetechnical Co Ltd, Tokyo, Japan), and snap-frozen in liquid nitrogen. Ten micron sections were mounted to polylysine slides (Merck Ltd, Poole, UK) and dried at 37 ºC for 2 h. The sections were then fixed in 4 % paraformaldehyde in PBS at 4 ºC for 10 min. The sections were washed twice with PBS and incubated in freshly prepared filtered X-gal buffer containing X-gal 1 g/L, $MgCl_2$ 2 mmol/L, $K_4Fe(CN)_6$ 5 mmol/L, $K_3Fe(CN)_6$ 5 mmol/L and 0.5 % Nonidet p-40 in PBS, pH 7.4, at 37 ºC overnight, followed by counterstaining with nuclear fast red.

Measurement of hFIX After DNA delivery, mouse blood was collected periodically, and the serum hFIX level was quantitated by ELISA[13].

Immunocytochemical examination of hFIX in the liver For immunohistochemical assay, liver was fixed in 4 % buffered paraformaldehyde 24 h after plasmid injection.

Liver toxicity assay To evaluate liver toxicity in mice that received plasmids or chloroquin, blood was collected at different time points via retro-orbital bleeding into one-tenth volume of 0.15 mol/L sodium citrate. Samples were analyzed for the presence of alanine aminotransferase (ALT), aspartate aminotransferase (AST). Histological examination was performed on formalin-fixed mouse liver sections prepared by routine histological staining (hematoxylin/eosin) procedures.

Satistical analysis Data were shown as mean±SD and analyzed by ANOVA test.

RESULTS

Study on chloroquine dosage Mice given 1000 μmol/L chloroquine had laboured breathing, bradcardia and peripheral hypothermia after injection, and disappeared after 12 h. Mice given 100, 200, and 500 μmol/L of chloroquine showed none of the above signals. For these reasons, we believed 1000 μmol/L or higher chloroquine was not acceptable for *in vivo* study.

Localization of β-galactosidase gene expression Recent reports related to hydrodynamics-based procedure indicated that the majority of DNA was ended up in liver and very low levels of vector DNA were observed in lung, spleen, and heart. To clarify the transgene expression site, we delivered 10 μg pCMVβ

and 200 μmol/L chloroquine in the tail vein. LacZ gene expression was assessed in various organs including the liver, heart, lung, kidney, spleen, muscle, stomach, brain, and spermary. X-gal only stained the liver of pCMVβ-injected mice (Fig 1), but it was negative in control mice. We did not find convincing examples of X-gal-stained cells in the heart, lung, kidney, spleen, muscle, stomach, brain and spermary. These results were confirmed by the other reports[14].

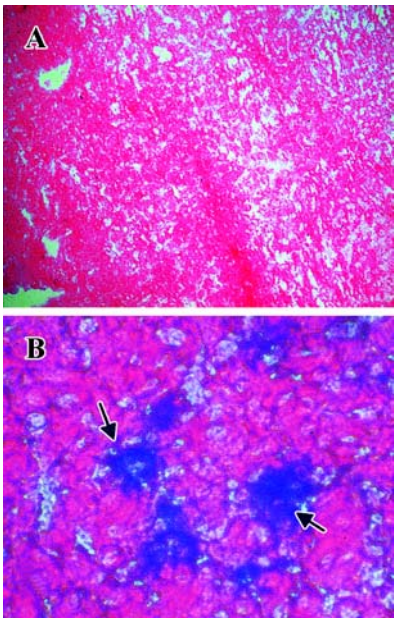

Fig 1. β-Galactosidase staining of livers. Mice given 10 μg pCMVβ and 200 μmol/L chloroquine in 2.2 mL of Ringer's solution or only Ringer's solution by the tail vein injections within 6-7 s. A) a liver section after injection of Ringer's solution, ×100; B) a liver section after plasmid with 200 μmol/L chloroquine injection (arrows showed the positive stains), ×400.

ELISA analysis of hFIX expression in the liver We examined the level and stability of hFIX expression *in vivo* after tail vein injection of 10 μg of pCMV- hFIX with chloroquine at different concentrations. The rapid injection of plasmid in 2.2 mL of Ringer's solution was completed within 6-7 s. The level of hFIX expression was examined by ELISA at different time points. As shown in Fig 2, the marked FIX levels linear enhancement (2-4 folds) was observed as the addition of chloroquine increased from 100 to 200 μmol/L compared to plasmid alone (P<0.01 for the first three days). The maximum gene expression was observed in the 200 μmol/L chloroquine-treated animals. Eight hours after injection, the average expression of hFIX in 200 μmol/L chloroquine-treated animals was 4.4±1.8 mg/L (n=9), it was 3 times higher than that of the control animals 1.3±1.6 mg/L (n=6). At 24 h, the hFIX level increased in 200 μmol/L chloroquine-treated mice to a concentration of 9.7±1.6 mg/L, it was 4 times higher than that of the control mice 2.4±0.5 mg/L. Nevertheless, the serum hFIX levels in 500 μmol/L chloroquine-treated mice were lower than that of the control in the first day. There is no significant difference among all the treated groups (P>0.05) 3 d later.

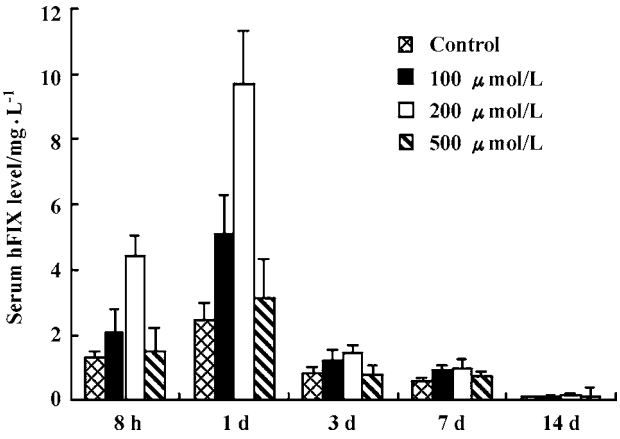

Fig 2. The concentration of hFIX in mice after the injection of 10 μg pCMV-hFIX and chloroquine. control, 10 μg pCMV-hFIX only; 100 μmol/L , 10 μg pCMV-hFIX and 100 μmol/L chloroquine; 200 μmol/L, 10 μg pCMV-hFIX and 200 μmol/L chloroquine; 500 μmol/L, 10 μg pCMV-hFIX and 500 μmol/L chloroquine.

Immunohistochemical analysis of hFIX expression in the liver To elucidate the population and location of cells in the liver that express the transgene, 10 μg of pCMV- hFIX plasmid DNA with 0, 100, 200, 500 μmol/L chloroquine was injected into mice respectively, 24 h later, liver cells expressing the hFIX gene were identified by immunostaining. Compared with control animals which received plasmid only (Fig 3B), positive cells and positive signals were easily observed in liver sections from animals injected with plasmid and 200 μmol/L chloroquine-treated animals (Fig 3C).

Liver damage and toxicity test The potential toxic effects of chloroquine on animals were assessed by two independent approaches. The first one employs the observation on the histologic sections of the liver. Compared with the control, the degree and pattern of tissue damage was similar with animals injected with chloroquine at different concentrations (Fig 4). Those phenomena suggested that the damage was not related to chloroquine, primarily caused by the fluid bolus[15].

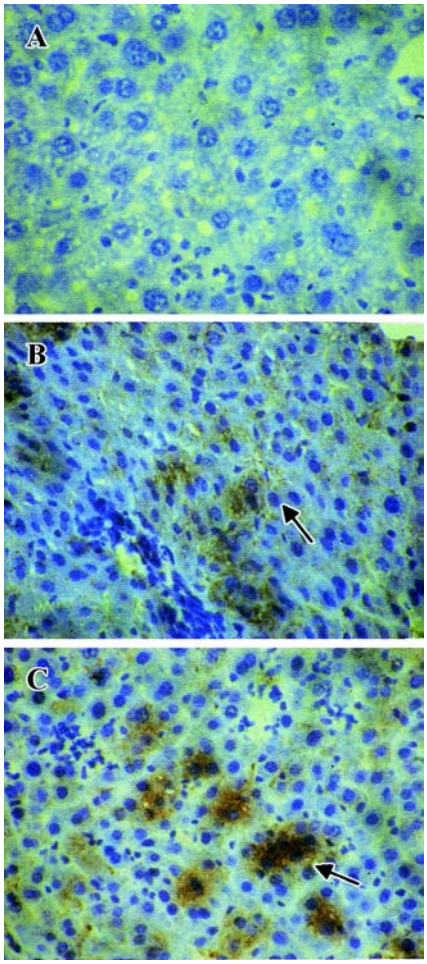

Fig 3. Immunohistological staining of the liver. Plasmid DNA 10 μg with chloroquine was injected into mice respectively. Twenty-four hours later, mice were killed and the liver sections were immunostained to detect hFIX expression. A) a liver section after injection of Ringer's solution; B) a liver section after plasmid injection; C) a liver section after plasmid with 200 μmol/L chloroquine injection (arrows showed the positive stains), ×100.

A clinical biochemical assay was also performed. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in sera from mice were assayed at 24 h, 3 d, 7 d, and 14 d after infusion. ALT level was elevated 40 folds and AST 3 folds above the normal on d 1 after injection and then rapidly declined to normal level 3 d and 7 d later (Tab 1). When mice sera were tested 14 d after injection, normal ALT and AST levels were detected from all the mice examined. It was noted that animals injected with plasmid only or plasmid containing chloroquine gave an identical pattern of ALT and AST changes. Among all the groups, there was no statistical difference, as analyzed by using one-way analysis of variance (ANOVA) test (P>0.05, data not shown), suggested that the increased ALT and AST value was supposed not induced by chloroquine.

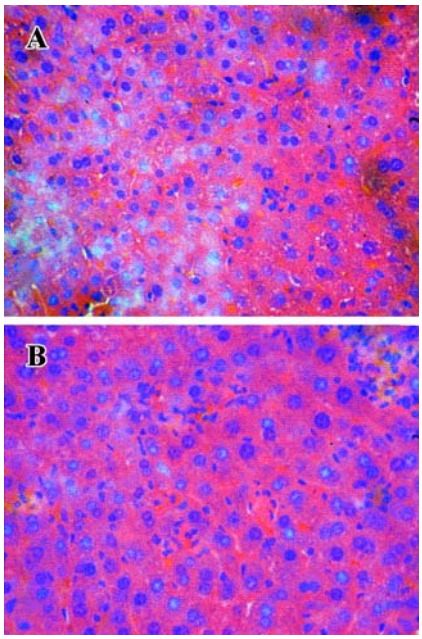

Fig 4. Hematoxylin/eosin staining of the liver sections. Mice were given chloroquine at different concentrations in 2.2 mL of Ringer's solution by the tail vein injections within 6-7 s. After 24 h, mice were killed and the liver sections as stained by a hematoxylin/eosin staining. Sections were made from mice received 0 (A), 200 μmol/L (B) of chloroquine, ×100.

DISCUSSION

Chloroquine is one of the most successful chemotherapeutic agents ever synthesized because of its safety, affordability, ease of use and its great efficacy. Reports showed the results of gene expression *in vivo* always did not correlate with that obtained from tissue culture[16]. Though there were reports of gene transfection studies in cell culture[12,13], but chloroquine has never previously been evaluated *in vivo*. Here we report the effect of chloroquine on exogenous gene expression in mice by hydrodynamics-based plasmid adminstration. Our results showed that concentration of chloroquine played an important role in the expression of hFIX. The maximum hFIX level 4.4±1.8 mg/L was obtained only in 200 μmol/L chloroquine-treated animals, 8 h after injection. After 24 h, hFIX expression level increased to a concentration of 9.7±1.6 mg/L, which is 3-4 fold higher than that of control. However, hFIX level in 500 μmol/L chloroquine-treated mice was lower than that of the control. Though the effects of

Tab 1. Biochemical assay for study of liver toxicity. CQ, chloroquine; ALT, alanine amino transferase; AST, aspartate amino transferase. *n*=6. Mean±SD. [a]*P*>0.05, [b]*P*<0.05 *vs* normal.

	d 1	d 3	d 7	d 14
ALT level/U·L^{-1}				
Normal	22±2			
Plasmid (control)	890±62	65±28	31±10	24±6
Plasmid and 100 μmol/L CQ treated	724±56[b]	28±15[a]	25±4[a]	25±4[a]
Plasmid and 200 μmol/L CQ treated	689±79[b]	28±18[a]	21±5[a]	23±8[a]
Plasmid and 500 μmol/L CQ treated	623±64[b]	24±10[a]	23±7[a]	20±3[a]
AST level/U·L^{-1}				
Normal	68±8			
Plasmid (control)	167±67	98±26	73±12	72±6
Plasmid and 100 μmol/L CQ treated	126±56[b]	78±39[a]	64±21[a]	67±11[a]
Plasmid and 200 μmol/L CQ treated	117±48[b]	67±31[a]	68±18[a]	64±9[a]
Plasmid and 500 μmol/L CQ treated	120±6[b]	70±33[a]	70±23[a]	65±7[a]

chloroquine on the gene transfer efficiency require further study, the chloroquine enhanced transfection efficiency was presumably mediated through its pH elevating effect on the lysosomal compartment, thereby, protecting DNA molecules from nuclease degradation. Chloroquine was also known to bind strongly to DNA and it might therefore protect DNA molecules from nuclease degradation[10]. In addition, reports also found that at higher concentration, chloroquine inhibited protein[17] and polypeptides synthesis[18] finally caused cell lysis[19].

In this study the level of hFIX expression reached its peak between 8 h and 24 d and then declined gradually. There would be few explanations for the decline of hFIX expression. Firstly, reduce of chloroquine concentration. Chen *et al* demonstrated that majority of DNA began to loss 1 d after injection and 78 % of DNA were lost 5 weeks later[3]. Our studies demonstrated a requirement for concentration of chloroquine for enhancement of gene expression. But because of half-life of chloroquine and metabolism *in vivo*, serum concentration of chloroquine could not keep steady *in vivo*. As decline of concentra-tion, gene transfer efficiency of chloroquine became less evident. Secondly, the anti-hFIX antibody induced *in vivo*. High level of hFIX expression would induce the production of antibody *in vivo*, which would clear away the cells expressing hFIX protein or eliminate hFIX proteins in plasma[3,15]. Finally, the modification of foreign gene by the self protection of cells would shut down the expression of hFIX[20]. The CMV promoter delivered *in vivo* was known to induce cytokine production and the cytokine-mediated effects subsequently attenuated the promoter activity and limited the transgene expression[21]. The structure of cDNA was also a critical reason causing declining of gene expression[22] though in our study, part of the introns were added in FIX cDNA. Recently, several strategies have been used to prolong gene expression, for example, by using chemical reagents to demethylate the foreign gene *in vitro*, immunodepressants to depress the induction of immunity, locus control region to prevent gene silencing, mammalian promoters to drive gene expressing, genomic structure to take place the cDNA structure of interested gene and so on.

Transaminase levels and liver histological study showed the damage of liver was not related to chloroquine, primarily caused by the fluid bolus.

These results demonstrated that chloroquine appeared to be an attractive agent to improve and sustain exogenous gene expression *in vivo*.

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