ORIGINAL RESEARCH

Role of L-Arginine on the Expression of Coagulation Factor VIII Gene

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ABSTRACT

Objective • To explore the key sites in which L-arginine affects the expression of human coagulation factor VIII gene, and to create new drug targets for the treatment of hemophilia.

Methods • A total of 5 human FVIII genes (A1, A2, A3, C1 and C2) with B domain deletion were transfected into human umbilical vein endothelial cells (HUVECs) as promoters. Run-on assay and ELISA analysis were performed to observe the driving effect of each domain gene on chloramphenicol acetyl transferase (CAT) gene transcription and expression, and the effect of L-arginine on each promoter.

Results • In co-culture with L-arginine, transcriptional expression of the CAT gene was not detected in the PCAT3-Basic group (negative control without promoters), PA3-CAT3-Enhancer group or PC1-CAT3-Enhancer group. The transcriptional expression of CAT gene in the PCAT3-Control group (positive control with promoters) and PA1-CAT3-Enhancer group was unchanged compared with the non-L-arginine intervention, while the transcriptional expression of CAT gene in the PA2-CAT3-Enhancer group was significantly enhanced.

Conclusions • A1 and A2 domain genes had promoter function and could initiate the transcription and expression of CAT gene, but A3, C1 and C2 domain genes could not. Moreover, L-arginine can significantly enhance transcription and expression of human coagulation factor VIII via A2 domain (*Altern Ther Health Med.* 2023;29(3):26-31).

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INTRODUCTION

Human coagulation factor VIII (FVIII) is a component of normal human plasma and plays an important role in blood coagulation. A defect in the FVIII gene can result in a blood clotting disorder called hemophilia A due to a deficiency or abnormal function of FVIII in plasma.[1-3] Hemophilia A is a common X chromosome-linked recessive hereditary hemorrhagic disease. The incidence of hemophilia A in the male population is approximately 1/5000 to 1/10000; 85% is severe.[4] The clinical manifestations of this disease are mainly spontaneous and traumatic bleeding of deep soft tissues (such as joints and muscles) and viscera.[5] Due to long-term bleeding, patients often develop joint malformations and become disabled, and viscera hemorrhage (especially cerebral hemorrhage) is often life-threatening.[5]

At present, the main method of treatment in hemophilia A is made from normal plasma FVIII concentrate on replacement therapy. The therapy can effectively control bleeding symptoms,[6] but is derived from the normal plasma FVIII concentrate of which is limited in availability. The other existing alternative treatments have many disadvantages and limitations, such as the spread of viral diseases like HIV/AIDS, hepatitis, etc.[7] In recent years, although recombinant human FVIII has been marketed, it has a high price tag due to its low expression level and short expression duration in the preparation process.[8] Thus, improving the expression level and prolonging the expression time of the FVIII gene by studying its expression regulation is the focus and direction

of current and future research in hemophilia A treatment. Our previous study found that L-arginine can enhance transcription and expression of human coagulation factor VIII gene *in vitro*, and this study further explored its mechanism of action.

MATERIALS AND METHODS

Materials

Human FVIII complementary DNA (cDNA) with deletion of the B domain (B domain deleted human prosthetic factor VIII complementary deoxyribonucleic acid, BDD FVIII cDNA) recombinant plasmid PCI-neo-BDDHFVIII was donated by Shanghai Institute of Hematology. Vectors PCAT3-Basic, PCAT3-Control and PCAT3-Enhancer were purchased from the Promega Company, Madison, Wisconsin USA; All restriction endonuclease and T4DNA ligase are products of TaKaRa Bioengineering Co., Ltd., Shiga, Japan; RPMI 1640 medium, TRIzol and diethyl pyrocarbonate were all purchased from GIBCO™, Grand Island, New York, USA; fetal bovine serum was purchased from HyClone Media, Logan, Utah USA; SuperFect transfection reagent was purchased from QIAGEN Sciences, Germantown, Maryland USA; agarose was purchased from AMRESCO, Cleveland, Ohio USA; A-32p-utp was purchased from Beijing Yahui Biomedical Engineering Company. Bio-dot wire printing device, vacuum dryer, 32P Sensitizer screen, Molecular Imager FX system and Type 680 Microplate were purchased from Bio-Rad Chemicals, Inc., Hercules, California USA; Syngene image analysis system was purchased from Syngene Corporation, Bangalore India; CAT test kit was purchased from Roche, Basel Switzerland.

Methods

Vector construction

Polymerase chain reaction (PCR) was used to amplify the A1, A2, A3, C1 and C2 genes of human FVIII cDNA with domain B deletion. The amplified primers and annealing temperature are shown in Table. KpnI restriction endonuclei restriction site was added to the 5' end of each upstream primer, and XhoI restriction endonuclei restriction site was added to the 5' end of each downstream primer. PCR amplification conditions: Pre-denaturation at 95°C for 5 min, then 94°C for 1 min, reaction at the corresponding annealing temperature of each primer for 1 min, and 72°C for 90 sec. After 35 cycles, extension at 72°C for 10 min. PCR products were detected by 1.2% agarose gel electrophoresis. The amplified A1, A2, A3, C1 and C2 gene fragments were recovered, purified and inserted into the site between the KpnI and XhoI of the reporter gene vector PCAT3-Enhancer containing CAT, respectively. A total of 5 vectors— PA1-CAT3-Enhancer, PA2-CAT3-Enhancer, PA3-CAT3-Enhancer, PC1-CAT3-Enhancer and PC2-CAT3-Enhancer— were constructed. The 5 vectors were digested by restriction endonuclides BalI, BamHI and ApaLI, and the 2 ends of the link sites were sequenced by using RVprimer3 and RVprimer4 primers on the 310 DNA-sequencer.

Isolation and culture of human umbilical vein endothelial cells (HUVECs)

HUVECs were isolated and collected by collagenase digestion under aseptic conditions. A 20-cm piece of umbilical cord was obtained from healthy pregnant women who delivered at term and umbilical cord cells were inoculated into 75-ml culture bottles covered with gelatin. They were placed in a 37°C incubator with 5% CO_2 for static cultivation. The medium consisted of 20% fetal bovine serum, endothelial cell growth supplement 50 ng/ mL, penicillin 100 U/mL and streptomycin RPMI 1640 100 mg/ml. After fusion, the culture of HUVECs were sub-cultured at a 1:3 ratio. The cells were typically monolayer paving stones and were positive for FVIII-associated antigen. The second generation HUVECs were used in the experiment.

Cell grouping, gene transfection and L-arginine induction

Cultured HUVECs were inoculated into 6-well culture plates (2.0×10^5 cells/well) and randomly divided into 14 groups; the PCAT3-Basic I group and PCAT3-Basic II group, PCAT3-Control I group and PCAT3-Control II group, PA1-CAT3-Enhancer I group and PA1-CAT3-Enhancer II group, PA2-CAT3-Enhancer I group and PA2-CAT3-Enhancer II group, PA3-CAT3-Enhancer I group and PA3-CAT3-Enhancer II group, PC1-CAT3-Enhancer I group and PC1-CAT3-Enhancer II group and PC2-CAT3-Enhancer I group and PC2-CAT3-Enhancer II group. Each group had 12 culture holes. PCAT3-Basic were used as a negative control, and PCAT3-Control were used as a positive control. All HUVECs in each group were cultured in a 37°C, 5% CO_2 incubator. When the cells were completely adhered to the wall and grew to 70% to 90% fusion,

Table. Amplified Primer for FVIII Domain Genes

Domain gene	Primer	Annealing temperature
A1	Upstream: 5'-TTTTGGTACCCGACCACCATGCAAATAG-3'	61.5 °C
	Downstream: 5'-AATCTCGAGTCATAGTCTTCCGCTTCTT-3'	
A2	Upstream: 5'-ACTGGGTACCAACCCCAACTACGAATGA-3'	60.5 °C
	Downstream: 5'-ATATCTCGAGCCTCTGTTCCGAAAGTCT-3'	
A3	Upstream: 5'-GTCCGGTACCGTTGAAATGAAGAAGGAA-3'	57.5 °C
	Downstream: 5'-AATCTCGAGCTGACACTTATTGCTGTA-3'	
C1	Upstream: 5'-TTAAGGTACCATGACTCCCCTGGGAATGGCT-3'	59 °C
	Downstream: 5'-TATACTCGAGTGGCATGCTGCAACTATT-3'	
C2	Upstream: 5'-ATTGGTACCATGTTGGGAATGGAGAGTAAAG-3'	60 °C
	Downstream: 5'-TTTTCTCGAGAGAAGGCAAGCCAGGGAG-3'	

the culture medium was discarded and washed with phosphate-buffered saline (PBS). Each group of HUVECs corresponded to PCAT3-Basic, PCAT3-Control, PA1-CAT3-Enhancer, PA2-CAT3-Enhancer, PA3- CAT3-Enhancer PC1-CAT3-Enhancer and PC2-CAT3 Enhancer. Each group of fusion HUVECs was digested with 0.25 % trypsin and inoculated into 6-well culture plates with approximately (2-4) × 10^5 cells per well. When the cells grew to 40% to 60% after 24h, the cell culture medium was removed, and the cells were washed with PBS. 5-mg plasmid vectors dissolved in 30 ml TE buffer (10 mmol Tris-HCl, 1 mmol EDTA, pH=8.0) were diluted to a total volume of 150 ml by using RPMI 1640 medium without serum and antibiotics. After mixing, 20 ml SuperFect Transfection reagent was added. The mixture was mixed again and placed at room temperature for 10 min to form a composite with the SuperFect Transfection reagent. 1 ml of RPMI 1640 medium containing serum and antibiotics was added to the complex, which was mixed and quickly added to the washed HUVECs. After incubating for 4 h in an incubator containing 5% CO_2 at 37°C, RPMI 1640 medium was abandoned and the cells were washed with PBS 4 times. After 24 h culture with fresh RPMI 1640 medium containing fetal bovine serum (FBS) (20% by volume), the PCAT3-Basic II group, PCAT3-Control II group, PA1-CAT3-Enhancer II group, PA2-CAT3-Enhancer II group, PA3-CAT3-Enhancer II group, PC1-CAT3-Enhancer II group and PC2-CAT3-Enhancer group were cultured with L-arginine (final concentration: 10 mmol/L) for 24 h. Then, the PCAT3-Basic I group, PCAT3-Control I group, PA1-CAT3-enhancer I group, PA2-CAT3-Enhancer I group, PA3-CAT3-Enhancer I group, PC1-CAT3-Enhancer I group and PC2-CAT3-Enhancer group I were added with an equal volume of RPMI 1640 medium.

Nuclear isolation by expansion lysis

HUVECs in each group after 48 culture were respectively scraped into pre-cooled PBS by latex curettes. After suspension with PBS, the cells were centrifuged for 1000 g × 5 min and washed. 4 ml RSB solution (10 mmol/l NaCl, 10 mmol/l Tris-HCl (pH = 7.4), 35 mmol/l $MgCl_2$) was added to the cell precipitates, which were placed on ice for 5 min to lysis in the hypoosmotic buffer. We added Triton X-100 with 10% volume fraction, mixed solution well and added 4 mol/l potassium chloride (KCl) to the final concentration of 140 mmol/l. The cells were homogenized 10 to 15 times in a Dounce manual homogenizer and a microscope was used to observe cell lysis and nuclear release. The nuclei were collected by centrifugation at 1000 g × 5 min. After washing with cold PBS, the precipitation was suspended with non-specific binding (NSB) solution [50 mmol/L HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH=8.0), 5 mmol/L $MgCl_2$, 0.5 mmol/L DTT (dithiothreitol), 1 mg/ml BSA (bovine serum albumin), 25% glycerol] and frozen in liquid nitrogen.

Run-on assay of *in vitro* nuclear

The nuclei in the NSB solution were thawed and centrifuged for 1000 g × 5 min to remove supernatant. The samples were suspended with 200 mL HRB buffer [20 mmol/L Tris-HCl (pH = 7.9), 140 mmol/L KCl, 5 mmol/L $MgCl_2$, 1 mmol/L DTT, 40U RNA inhibitor, 1 mmol/L ATPm, 1 mmol/L CTP, 1 mmol/L GTP, 1 mCi -32P-UTP]. The suspension was transferred to 1.5 mL Eppendorf centrifuge tube and placed in 37°C water bath for 30 min for nuclear transcription reaction. At the end of incubation, 1 mL TRIzol was added to extract 32P-RNA transcribed from the nucleus.

Bio-dot hybridization

CAT gene fragment was used as a probe and a 452-bp cDNA fragment of human glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal reference. Sodium hydroxide (NaOH) was added to the DNA solutions containing 5 mg CAT gene fragment and 5 mg GAPDH cDNA fragment to the final concentration of 0.1 mol/l. After mixing, the DNA fragments were placed at room temperature for 30 min to denature. An equal volume of 3 mmol/L ammonium acetate was added to neutralize. The nitrocellulose membrane (NC membrane) was loaded into the Bio-dot device and divided into 10 columns of adding sample wells, and each column was divided into 2 rows. The denatured CAT gene fragment and GAPDH cDNA fragment were added, and the sample wells were vacuum dried. The sample wells were washed twice with 0.5 mL 6´SSC (1 mol/l NaCl, 0.1 mol trisodium citrate, pH = 7.0). The NC membrane was taken out and dried in a vacuum dryer, and the 2 DNA fragments were crosslinked on the NC membrane. The NC membrane was divided into 10 sections. 15 mg labeled 32P HUVECs nuclear transcription RNA was hybridized with CAT gene fragment and GAPDH cDNA fragment on NC membrane.

The images were developed using a 32P-enhanced screen and observed by Molecular Imager FX system. Image scanning analysis was performed by Syngene image analysis system to determine the transcriptional intensity of CAT gene in the absence of domain B was determined when the 5 domains of human FVIII cDNA genes A1, A2, A3, C1 and C2 were used as promoters.

Detection of CAT content

CAT content in HUVECs lysates was determined by enzyme-linked immunosorbent assay (ELISA). The medium of HUVECs was removed and washed with 5 ml cold PBS 3 times. 1 ml cell lysate was added and the solution was lysed at 20°C for 30 min. Cell extracts were frozen at -70°C for ELISA. The detection procedure was carried out according to the CAT ELISA kit instructions. A Bio-Rad 680 microplate analyzer was used to detect the absorbance A value at 415 nm and 490 nm, and the content of CAT in cell extracts was determined according to the standard curve.

Statistical Analysis

Data are expressed as mean ± standard information (SD). An analysis of variance (ANOVA) was used to determine the difference. Statistical analyses were performed via IBM® SPSS 25.0 software. A *P* value <.01 was considered statistically significant.

RESULTS

Identification of Recombinant Plasmid Vectors

Recombinant plasmid vectors of PA1-CAT3-Enhancer, PA2-CAT3-Enhancer, PA3-CAT3-Enhancer, PC1-CAT3-Enhancer and PC2-CAT3-Enhancer were identified by digestion of restriction endonucleases BalI, BamHI and ApaLI enzymes and sequencing identification, indicating that the construction was correct.

Effects of L-arginine on CAT Gene Transcription in Each Group (Run-on Assay)

Transcriptional expression of CAT gene between non-L-arginine and L-arginine in the PCAT3-Basic group (negative control without promoters), PCAT3-Control group (positive control with promoters), PA1-CAT3-Enhancer group, PA2-CAT3-Enhancer group, PA3-CAT3-Enhancer group, PC1-CAT3-Enhancer group and PC2-CAT3-Enhancer group were 6.46 ± 0.56 vs 6.44 ± 0.64 pg/10^6 cells, 724.33 ± 31.16 vs 720.19 ± 31.39 pg/10^6 cells, 61.85 ± 7.86 vs. 61.79 ± 7.97 pg/10^6 cells, 60.27 ± 7.27 vs 368.56 ± 21.85 pg/10^6 cells, 6.46 ± 0.70 vs 6.44 ± 0.57 pg/10^6 cells, 6.46 ± 0.54 vs. 6.48 ± 0.84 pg/10^6 cells and 6.50 ± 0.82 vs 6.44 ± 0.78 pg/10^6 cells. Run-on analysis showed that in the absence of L-arginine intervention, no transcriptional expression of CAT gene was detected in PCAT3-Basic group (negative control without promoters), and there was strong transcriptional expression of CAT gene in the PCAT3-Control group (positive control with promoters). Weak transcriptional expression of CAT gene was detected in PA1-CAT3-Enhancer group and PA2-CAT3-Enhancer group. The transcriptional expression of CAT gene was not detected in the PA3-CAT3-Enhancer group, PC1-CAT3-Enhancer group or PC2-CAT3-Enhancer group. However, in the co-culture with L-arginine, the transcriptional expression of CAT gene was not detected in the PCAT3-Basic group (negative control without promoters), PA3-CAT3-Enhancer group and PC1-CAT3-Enhancer group. The transcriptional expression of CAT gene in the PCAT3-Control group (positive control with promoters) and PA1-CAT3-Enhancer group was unchanged compared with non-L-arginine intervention, while the transcriptional expression of CAT gene in PA2-CAT3-Enhancer group was significantly enhanced (Figure 1).

Figure 1. Effects of L-arginine on CAT gene transcriptional expression in different HUVECs groups (run-on assay).

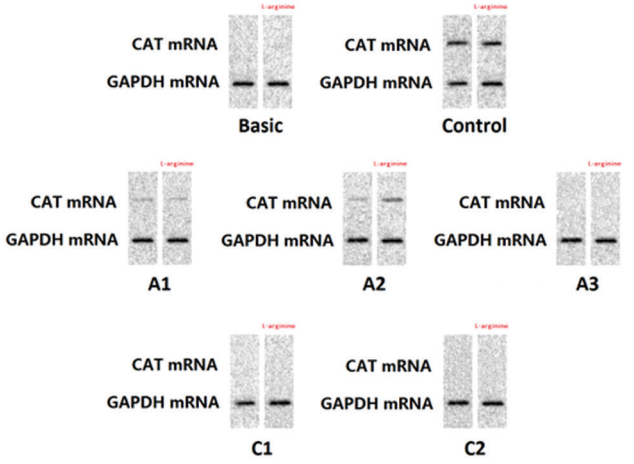
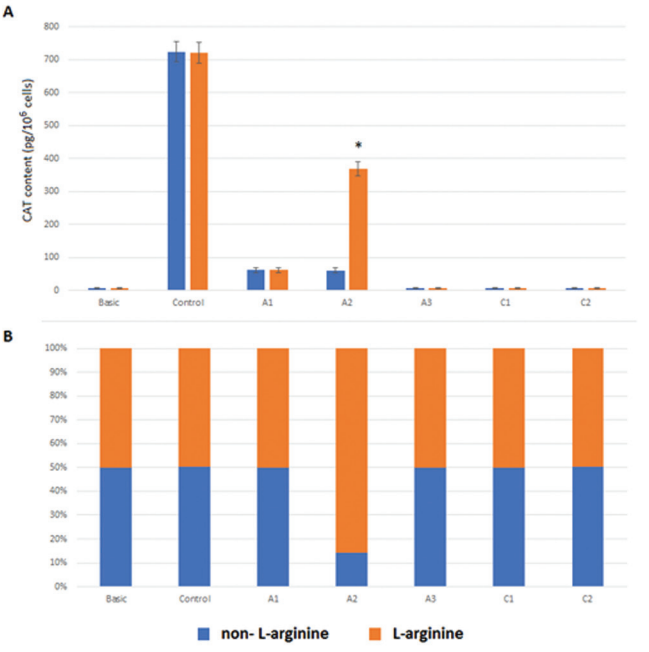


Figure 2. Effects of L-arginine on CAT gene transcriptional expression in different HUVEC groups (quantitative validation).



Effects of L-arginine on CAT Gene Transcription in Each Group (Quantitative Validation)

ELISA analysis showed that in the absence of L-arginine intervention, transcriptional expression of CAT gene in the PCAT3-Basic group (negative control without promoters), PCAT3-Control group (positive control with promoters), PA1-CAT3-Enhancer group, PA2-CAT3-Enhancer group, PA3-CAT3-Enhancer group, PC1-CAT3-Enhancer group and PC2-CAT3-Enhancer group were (6.46 ± 0.56) pg/10^6 cells, (724.33 ± 31.16) pg/10^6 cells, (61.85 ± 7.86) pg/10^6 cells, (60.27 ± 7.27) pg/10^6 cells, (6.46 ± 0.70) pg/10^6 cells, (6.46 ± 0.54) pg/10^6 cells and (6.50 ± 0.82) pg/10^6 cells, respectively. However, in the co-culture with L-arginine, transcriptional expression of CAT gene in the PCAT3-Basic group (negative control without promoters), PCAT3-Control group (positive control with promoters), PA1-CAT3-Enhancer group, PA2-CAT3-Enhancer group, PA3-CAT3-Enhancer group, PC1-CAT3-Enhancer group and PC2-CAT3-Enhancer group were (6.44 ± 0.64) pg/10^6 cells, (720.19 ± 31.39) pg/10^6 cells, (61.79 ± 7.97) pg/10^6 cells, (368.56 ± 21.85) pg/10^6 cells, (6.44 ± 0.57) pg/10^6 cells, (6.48 ± .84) pg/10^6 cells and (6.44 ± .78) pg/10^6 cells, respectively (Figure 2).

DISCUSSION

The traditional treatment for hemophilia A is desmopressin, which is used to combat dual defects: the primary defect (von Willebrand factor defect) and secondary

defect (Factor VIII coagulant activity defect). The goal of gene therapy in hemophilia A is to regulate the expression of the FVIII gene. At present, the common problem faced by gene therapy research in hemophilia A is the low expression level of the FVIII gene or the short expression duration. How to improve the expression level of the FVIII gene and prolong the expression time is an important part of the study of FVIII gene expression regulation. The regulation mechanism of FVIII gene expression is extremely complex, involving pre-transcriptional, transcriptional and post-transcriptional splicing, as well as protein translation and modification.[9,10] Human FVIII protein is divided into A1, A2, B, A3, C1 and C2 domains. The sequence is A1-A2-B-A3-C1-C2. Each domain is encoded by corresponding domain genes, among which A1-A2-B domain genes encode the heavy chain of FVIII, and A3-C1-C2 domain genes encode the light chain.[11] In our study, FVIII cDNA with B domain deletion was used, whose heavy chain genes only included A1-A2 domain genes and light chain genes included A3-C1-C2 domain genes.[11] PCAT3-Basic in this study had no eukaryotic promoter and enhancer and was used as a negative control. PCAT3-Control contained an early promoter of SV40, which can drive the transcription and expression of CAT gene, and an early enhancer of SV40, which is located at the 3' end of the CAT gene and was used as a positive control in this study. PCAT3-Enhancer does not contain promoters, but early enhancers of SV40 are located at the 3' end of CAT gene.

In our study, 5 human FVIII genes with B domain deletion were transfected into HUVECs as promoters, to observe the driving effect of each domain gene on CAT gene transcription and expression, and the effect of L-arginine on each promoter. Via *in vitro* run-on assay and ELISA analysis, it was observed that both A1 and A2 domain genes had promoter function and could initiate the transcription and expression of CAT gene, but A3, C1 and C2 domain genes could not. Moreover, L-arginine can significantly enhance transcription and expression of CAT gene via A2 domain.

Nitric oxide (NO) is an ubiquitous mediator, and L-arginine is the precursor of NO production.[12] In the presence of NO synthetase (NOS), L-arginine produces NO and L-guanidine.[12] In recent years, the effects of L-arginine on the human body have attracted extensive attention. Oral L-arginine has been shown to be a novel approach in the treatment of hypertension due to its association with NO production.[13] Another clinical trial indicated that L-arginine may be useful for therapeutic and clinical purposes in patients with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes.[14] In addition, dysregulation of L-arginine metabolism has been confirmed in patients with severe asthma.[15] However, there are no reports concerning the application of L-arginine in the treatment of hemophilia A at present. Hemophilia A is a type of disease characterized by low expression of FVIII level and high risk for bleeding.[16] In our study, L-arginine was found to significantly enhance transcription and expression of FVIII via A2 domain. Of note, the potential function of FVIII A2 domain may be similar to that of the promoter region. A study has found that in patients with hemophilia, inhibitory antibody formation was associated with defects in the FVIII gene, in which genetic determinants in the promoter region can lead to an increased risk for inhibitor formation, but the specific mechanism was not clear.[17] An animal study showed that injection of vector containing normal donor DNA into the FVIII promoter knockout mouse model could restore FVIII expression.[18]

In addition, the A2 domain may be related to the hydrolysis stability of FVIII protein.[19] The active stable FVIII molecule was generated by eliminating charged residues at the a1-A2 and A2-A3 interfaces.[20] Leong, et al. proved that non-covalent stability of FVIII A2 domain can prolong its activity, leading to the enhancement of blood clot formation in hemophilia according to animal experiments.[20] Therefore, we hypothesized that by integrating the promoter coding sequence, the defective gene could be rescued and the patient could be relieved by therapeutic treatment. This research was consistent with our findings that the A1 and A2 domain genes of human FVIII can act as promoters to drive transcription and expression of the CAT gene. In addition, we found that L-arginine was an important switch for activating the A2 domain.

CONCLUSION

To sum up, we concluded that A1 and A2 domain genes had promoter function and could initiate the transcription and expression of the CAT gene, but A3, C1 and C2 domain genes could not. Moreover, L-arginine can significantly enhance transcription and expression of human coagulation factor VIII via A2 domain. Thus, L-arginine has the potential to be an advanced treatment strategy in hemophilia A.

FUNDING

None.

CONFLICT OF INTEREST

None.

Ethics Approval

The study protocols were conducted according to the principles of the Declaration of Helsinki and were approved by the Scientific and Medical Ethical Committee of the Second Affiliated Hospital of Shantou University Medical College.

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