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ORIGINAL ARTICLE

Polyethyleneimine Enhances. situ Gene Delivery, Expression. Skeletal Muscles. BALB/c mice. Pluronic-mediated System

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Abstract: Objective. correctly introduce cationic polyethyleneimine(PEI). Pluronic L64-mediated gene de-livery system. mice skeletal muscle. enhance. efficiency. gene expression. methods. studyDifferent PEI samples. transferred. mice tibialis anterior muscles via intramuscular injection. SalinePlasmid DNA(PDNA)And this may contribute to the process of gene de-livery in skeleton muscle-Based System,Although pDNA was not fully compressed in L/P/D-0. 5 group. Conclusion The L/P/D-0. 5 system can safely and effectively improved the expression of existing genes in skeleton muscle.

Keywords: BALB/c mice;Skeleton Muscle gene delivery;Polyethyleneimine;Pluronic

Skeletal Muscle Distribution,The gene encoding functional protein is transferred to skeletal muscle cells by intramuscular injection."Factory"In the patient"Production"Therapeutic protein, achieving individual,Precision therapy is a significant and promising treatment strategy (Lu *et al*.,2003a).How to safely and efficiently transfer foreign genes into muscle cells is the key to the success of this strategy..Although the plasmidDNA(PDNA)Can be transferred to muscle cells for expression, but due to direct injectionPDNAEasily degraded,Transfection efficiency is not ideal due to the low efficiency.Therefore, people have been devoted to developing various gene transfer strategies to improve the efficiency of Gene Transfer and Expression in muscle, such as e-transfer method.,Ultrasound and carrier mediated methods(MIR *et al*.1998;Gu, Rin2000;Lauritzen *et al*.2002;Lu *et al*.2003b;Wells2004;Lee *et al*.2012).This paper explore the cationic polymer---Polyethylenimine(PolyethyleneiminePEI)As an gene carrier in skeletal muscle in situ Gene Transfer System in application of possibility usePEI/pDNAComplex andPluronic L64Combined with construction the Security efficient of Gene Transfer System (L/P/D)Which,LSPluronic L64 PRepresentative PEIDRepresentative PDNA.By the Different Nitrogen and Phosphorus (N/P)OfL/P/DSystem mediated-of gene expression efficiency and in biological safety evaluation revealN/PAnd muscle in gene transfection efficiency of relationship and establishedPEIIn skeletal muscle in application of effective programme.The programme for screening and Development efficient,For use with clinical treatment of skeletal muscle in situ gene transfer system provide the appropriate of principle and Strategy.

1. Material and Methods

1.1 Experimental Animal

5~7/Weeks age body qualityNatural 20~25gOfBALB/cMale Mice some purchased in Chengdu da shuo biological science and technology limited the company [Experimental Animal Production License number: Syxk (Sichuan) 2015-030].Animal Experiment were in Sichuan University ethics committee recognized under the in

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accordance with the relevant regulations and system [experimental animal "with license number: Syxk (Sichuan) 2013-017].Will mice in21,Humidity45%~65%Of environment in random cage feeding.Every group6Only all mice were injection bilateral leg Tibialis Anterior Muscle.

1.2 Reagent and Instrument

A of polyethyleni mine25 kDa,Pluronic L64(Sigma-AldrichAmerican);β-Galactosidase(PCMV-LacZ)In situ Staining Kit (PIK Wan days,China);E.Z.N..TMPlasmid small extraction kit,PurelinkTMHigh purity plasmid a lot extraction kit

β-Galactosidase, Fluorescein enzyme (PCMV-Luc)And far-infrared fluorescence protein (PCMV-E2)Of3A plasmid by this laboratory Liu yi li Dr. provide.Chemidoctm XRS +Gel imaging system (Bio-RAdAmerican);Varioskan FlashMulti-function microplate reader (ThermoAmerican); Nanos ZEN 1600Style nano-particle size and potential analyzer MalvenBritish); NanoDrop 2000(ThermoAmerican); MFP-3D-BIOTMAtomic Force Microscope (Bruker American); - Vivo Imaging System(CRIAmerican).

1.3 Experimental Methods

1.3.1 Plasmid of preparation and IdentificationAll plasmid in Escherichia coli

Escherichia coli DH5αIn transformation amplification with the above mentioned Plasmid extraction kit extraction get.Preparation good plasmidNano-drop 2000Determination of concentration and purity,1%Agarose gel electrophoresis identification in-Natural 20Short-term save,-80Long-term save.

1.3.2 Muscle in situ injection operation preparation the experimental group samples concentration2 mg in mL^{-1}OfPDNASolution and concent ration PEIWorking Fluid by corre sponding N/P Mixed which,DNA Total Qualit MuGIncubated at room temperature Natural 20min After for mation N/PR espectively 010 Of PEI/pDNAComplex.Then take different proportion of complex and 10MuL 0. 4%(W/v)Pluronic L64Mixed with physiological saline dilution 40MuLPluronic L64Final concentration0. 1%

(W/v)Mixture at room temperature static 5min FormationL/P/DSystem(L/P/D-0. 5,L/P/D-3,L/P/D-10) Which L/P/D-0. 5Group representativePEI And PDNA Of N/P = 0. 5L/P/D-3Group N/P = 3L/P/D-10GroupN/P = 10.To physiological saline group for negative control group,PDNAGroup for positive control group 1Pluronic L64/pDNA Mixture (L/D) Group for positive control group2.

Mice7/dAdaptation period after its on both sides of the Tibialis anterior muscle were was Hair Removal, Disinfection. With syringe respectively learn 40MuL Control group solution and experimental Solution of the Group.Muscle injection along the parallel muscle fiber orientation needle about 2mm2~5 SComplete Injection.

1.3.3 Report gene detectionThe3A report gene detection system respectively from qualitative,Quantitative and gene expression evaluation of sustainabilityL/P/DSystem:

(1)β-Galactosidase qualitative detection:The organization to in situ staining of style the detection by observe the coloring range and color depth sentenced Broken gene expression situation.In accordanceβ-Galactosidase in situ Staining Kit instructions experimental including in ice in staining Stationary Liquid Solid

SetNatural 20 minWith phosphate buffer (PBS)Washing3Times after fixed of sample the staining Processing2 hMore than finally with digital camera collection of Image.

(2)Fluorescein enzyme quantitative detection:TheLuciferaseREporter Assay KitKit the detection.Specific steps including:Samples by ice uniform Pulp,-80Night cracking and4 12 000 r in min^{-1} Centrifugal3 minAfter takeNatural 20MuLSupernatant in don't light white96Hole ELISA plate in with multi-function microplate reader automatic and-like and read absorption value.At the same timeBCA Protein Assay KitThe sample content determination homogenization Processing.Standard and sample with microplate reader DeterminationOD_{562}Under the absorption value according to standard curve equation calculation the samples in protein content "with relative fluorescein enzyme activity (RLU/mg Protein)Said results.

3)Far-infrared fluorescence protein detection gene continuous expression situation: the living Imaging System of

muscle in situ injection after the first7/,14Days of red fluorescence proteinE2-CrimsonThe expression of the detection.Detection before mice Tibialis anterior muscle hair removal processing with yellow light excitation in600~

NmThe scanning fluorescence signal finally with instrument comes with software the fluorescence imaging analysis.

1.3.4 Organization pathology DetectionThe injection after the muscle organization and main organ (heart,Liver,Spleen,Lung,Renal) the tissue sections of and observe the evaluation cationic andPluronicCombined with the gene transfer system of biological safety for clinical "with provide basis.Muscle injection7/dAfter separation calf Tibialis anterior muscle or tail vein injection4 dAfter anatomy out mice main organPBSWashing after4%(W/v)More pom Stationary Liquid in fixed48 hAfter in ethanol and xylene solution in the gradient dehydration.Dehydration after of samples after paraffin embedding,Slice,Hematoxylin-Eosin (HE)Staining processing after with optical microscope.

1.3.5 L/P/D-0. 5System inPEI/pDNAComplex of Particle Size,Potential and Morphology Characterization 3MuGOr Natural 20MuG pDNAAnd concentration

Ng in ·MuL^{-1}PEIWorking Fluid by correspondingN/PMixed incubated at room temperature

MinAfterMilliQWater dilution1 mL.Will1 mLSample solution1 cmQuartz Cuvette into Malvern nano-particle size and potentialAnalyzer(Zeta-SizerMalvern)In by dynamic light scattering method

Dynamic light scatteringDLS)OkayN/PFor0. 5An arcanePEI/pDNAComplex of Particle Size,Dispersion and surface potential the detection SamplesDNAFinal concentration3MuG In mL^{-1}Each sample repeat3Times.

Will complex adsorption in mica on and dry after "with atomic force microscope (AFM)Characterization its particle size and morphology characteristics SamplesDNAFinal concentrationNatural 20MuG In mL^{-1}.

1.4 Statistical Analysis

TheOrigin,Prism5. 0The data statistical analysis the group between single factors variance (One-way ANOVA) OrTTest Analysis,P<0. 05For difference have statistical significance.

2. Results

2.1 β-Galactosidase qualitative and Fluorescein enzyme Quantitative Analysis

The is application cationic material construction efficient of skeletal muscle in situ Gene Transfer System of key.Muscle injection7/dAfter inβ-Galactosidase (Figure1:A) And fluorescein Enzyme(Figure1:B)Of expression level on,L/P/D-0. 5The gene expression level of the group was significantly higher than that of the positive control group.1.Positive control group2. However,N/P (L/P/D-3AndL/P/D-10) Gene expression began to decline significantly.Numerically, L/P/D-0. 5Group-mediated luciferase expression was positive in the control group.1.28. 6Times,Positive control group2.Of2. 5Times.However, when N/PAdd3. And 10The expression of foreign genes was almost undetectable, and its value was reduced by at least4.Order of magnitude.These results indicate that only at a specific lowN/PTime,PeiTo the system

Gene, inheritance, delivery and expression, yield, product, extreme shadow, sound.Therefore L/P/D-0. 5For OptimizationL/P/dSystem.

2.2 Expression of Fluorescent Protein Gene

Continuous Study of Exogenous Gene Expression in vivo is more convincing for assessing the intensity and persistence of gene expression.(Pu *et al.*,2014).In vivo imaging, positive controls1.AndL/P/D-0. 5Groups of Fluorescence signals can continue to express at least2.Zhou Jian

Apparent Attenuation.L/P/D-0. 5The fluorescence signal of the group was significantly higher than that of the positive control group.1.(Figure2.).

2.3 Histological Analysis

Intramuscular injection7 dAfter,L/P/D-0. 5Group and negative control groupHeNo tissue lesions were found in the

stained sections (Figure3.:A,B), Note less

QuantityPeiIt is safe for skeletal muscle in situ Gene Transfer System.WithN/PIncrease,L/P/D-3The group showed pathological changes such as muscle fiber degeneration and inflammatory cell infiltration (Figure3.:C). WhenN/PReach 10Time, HeLarge infiltration of inflammatory cells in stained sections,Pathological Changes of lymphocyte infiltration and muscle fiber necrosis (Figure3.:D).

Tail vein injection4 dAfter that, the acute toxicity of the system was evaluated by observing the tissue sections of the main organs..L/P/D-0. 5The results of the group were consistent with that of the negative control group (Figure4.)That is consistent with the results of local injection.

/P/D-0. 5It will not cause organ lesions, which further indicates that the system has good biocompatibility..

A. negative control group,B .l./P/D-0. 5 Group,C.l./P/D-3 Group,D. L/P/D-10 Group;The yellow arrow shows

2.4 L/P/D-0. 5Characterization of complexes in the system[304 Nm ± 7. 6 nm, Multiple dispersion coefficient (Polydisexcellence in- N/PFor0. 5OfPEI/pDNAComplexes (PEI/pDNA-Dex,PDI)= 0. 43], AndZetaThe potential is negative (-15. 9 ev± 0. 5)The particle size is shown in Fig.5.:AThe proportional complex has nano-size1. 4 EV)Show whenN/PFor0. 5Time,PeiWithPDNAFormation

3. Discussion

Skeletal Muscle in situ Gene Transfer and Expression System for gene treatment provide the ideal of strategy the system of open allow will many kinds of methods combined with Application to get better of treatment effect.Non-ionic Style MaterialPluronicCan improve the gene transfer efficiency main :(1)And Virus Carrier the to improve its safety and enhanced Gene Transfection Efficiency

Feldman *et al*.1997;Dishart *et al*.2003);(2)As an carrier modified Group(Jeon *et al*.2003)Or auxiliary agent(Astafieva

Et al.1996)Improve cationic non-Virus Vector in Serum/Physiological environment in stability and improve the gene transfection efficiency ;(3)Separate or and physical methods combined with can significantly enhance exogenous gene in muscle in situ Gene Transfer System in transfer/Expression Level (Gu, Rin2000;Song *et al*.2013;Liu *et al*.2014).PluronicMay through many kinds of Molecular Mechanism promote exogenous gene transfer and expression such as by enhanced cell membrane permeability to enhancePDNA,Virus Carrier or cationic Material/DNAComplex of uptake(Gebhart *et al*.2002);Promoting

PDNAIn skeletal muscle in penetration,Distribution or into nuclear role and(Gu, Rin2000;Pitard *et al*.2002).In a kindPluronic L64Mediated-of efficient of skeletal muscle in gene transfer programme in,Plu-ronic L64Use similar of phospholipid molecular structure and cell membrane each other role interference its integrity improve Membrane Permeability(Liu *et al*.

2014).However in the system in due to itsPluronic L64Don't andPDNAEach other role not protectionDNAIn complex of in Environment in easy to be nuclease degradation difficult to smooth,Security transfer to target Cell in the transfer great to reduce the muscle in Gene Transfer Efficiency and serious of organization inflammation (Pitard *et al*.2004;Burke&Pum2008).It is suggested that with negative charge or electrically neutral of material/PDNA

Complex can avoid andECMIn electronegativity molecular of non-specific binding may be suitable for muscle in Gene Transfer System.InPEIAndPDNAThe composite system in by regulation cationicPEIAndPDNAOf Proportion preparation the with different surface charge of Complex.So this research hope find a rightPEIAndPdnasOf proportion to formation overall with negative charge of complex to avoid andECMIn with negative charge molecular of each other role at the same time reducePEIThe amount of also can reduce even avoid local inflammation reaction and these all may be in favor of the objective gene to muscle cells in the transfer and Expression.This experimental results confirmed that onlyL/P/D-0. 5The Group can significantly increase the expression level of foreign genes, and maintain the high level of expression within the monitoring time.N/PIncrease,L/P/dThe gene expression was dramatically reduced, even lower than the positive control group.1..At the same time, whether it is intramuscular injection or tail vein

injection,L/P/D-0. 5They all show good biological compatibility.

It can be safely applied to skeletal muscle gene transfer to enhance the expression level of exogenous genes, and improveN/PMuscle tissue will have varying degrees of pathological changes, especiallyN/PTime (N/P = 10).The above-mentioned gene expression levels and biological safety data are consistent with the expected results, proving that in certain low-profile conditions,PEI/pDNAThe electronegativity of the complex indeed helps to construct efficient and safe in situ Gene Transfer in skeletal muscle/Expression System.It is worth mentioning that this proportion is completely invalid in vitro gene transfection experiments,N/PSite (Schmidtwolf&Schmidtwolf,2003). Meanwhile, In 10~15 Optimal transfection Condition.This also indicates that

Compared to nucleic acid molecules compressed into nanoscale, uncompressedBecause the conditions required for delivery are very different, this may be related

PDNADue to the loose molecular chain state, the efficiency of transmembrane Movement Different Environment.

Will decrease (Godbey *et al*.,1999).

A common nucleic acid protection strategy is to use cationic carriers to compress and protect negatively charged nucleic acid molecules through charge effect..As a recognized nucleic acid carrier, Branched Polyethylene imine is widely used in Gene Transfection in vitro, showing excellent transfection effect.(Xu *et al*.,2009;He *et al*.,2013).However, most in vivo experiments reveal the ineffective and even strong inhibitory effects of cationic materials on Gene Transfer and Expression in muscle.(TROs *et al*.,2010;Song *et al*.,2013;Pu *et al*.,2014).This may be due to the difference between the in vitro cell culture environment and the complex in vivo muscle environment (RUponen *et al*.,1999).Extracellular Matrix(Extracellular Matrix,ECM)A large number of negatively charged molecules can be combined with positively charged molecules.PEI/pDNAComplex, thereby interfering with the compound to the muscl

BecausePeiFull compressionDNATimeN/PFor3.(Dai *et al*.,2011;Dai&Wu,2012)So,N/PFor0. 5When, in the compoundDNACan't be completely compressed, but a small amountPeiThe additionDNAThe structure of the nanocomposites is relatively close, forming a certain three-dimensional shape and negatively charged.Although2.The particle size of the composites detected by different detection methods is different, and the error may be caused by different sample preparation conditions..AFMThe images reflect the 3D structure of the dried nanoparticles, whileDLSNanoparticles need to be measured in solution state(Li *et al*.,2001).We speculate that inL/P/D-0. 5In the system,PEI/pDNAThe electronegativity of the complex prevents it from being strandedECMIt is conducive to its transmission to the cells;2)

Et al.,2015)Creating favorable conditions for complex molecules to enter cells;PeiCompressionPDNAIt forms a more compact complex molecule, which is more conducive to its passage through the permeability increased cell membrane and resist the degradation of nuclease..These conditions makeL/P/D-0. 5The transfer and expression efficiency of the system exceeded that of plasmid andPluronic l64The system of mediation has reached a new height..

In this studyBALB/cMice as models, validate an important concept:WillPDNACompressed into negatively charged nano-composite particles, which can be applied and improvedPluronic l64In situ Gene Transfer and Expression in skeletal muscle.How to promote the proof of this concept?DNA/Material complexes provide a solution to enter skeletal muscle cells, creating more efficient in-situ Gene Transfer/The expression system will play a driving role in the design of a more appropriate material for molecular compressionDNATo further improve the expression level of foreign genes, and may even push the system to the application level..In fact, in this study, the persistent high expression of red fluorescent protein in mouse skeletal muscle cells after one-time intramuscular injection has shown that the system has,Application Prospect of chronic diseases.

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