Original Article

β-thalassemia for translating human β-globin gene and RNA interference of α-globin gene expression by lentivirus vectors

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Received April 14, 2016; Accepted August 26, 2016; Epub October 1, 2016; Published October 15, 2016

Abstract: Objective: According to the characteristics of α/β-globin gene imbalance caused by α-globin gene accumulation in β-thalassemia, we designed two lentiviral vectors, that is, while we used the lentiviral to mediate full length human β-globin gene, we introduced the interference fragment of lentiviral α-globin gene to adjust the α/β chain ratio by increasing the expression of β-globin and decreasing the expression of α-globin. Methods: We gained lentiviral vectors LV-β-globin by extracting DNA of β-globin expresser. According to the design principles of RNA interference sequence, the optimal dynamic parameters of RNA interference target sequence are selected based on the design experience and software evaluation. After lentivirals were stably transfected into K562 cells, the total RNA was extracted, and the expression of m RNA of α-(HbA2) and β-(HBB) globin gene in K562 cells was detected by PCR real-time. Western blotting was used to detect the expression of alhpa and β-globin. Results: β-globin gene was amplified to specific band of 227 bp by RT-PCR, and the expression of RT-PCR α-globin gene was amplified to specific band of 85 bp as well. 2⁻ΔΔCt value of β-globin gene mRNA was 4.080 ± 0.078 and 2⁻ΔΔCt value of α-globin gene mRNA was 0.274 ± 0.023. Results of western blotting test indicated that in the stably transfected K562 cells, the synthesis of human β-globin was significantly higher than that of the blank group and negative control group and the synthesis of α-globin was significantly lower than that of the blank group and negative control group. Western blotting showed that the grey level of β-globin was 1.063 ± 0.082, and the grey level of α-globin was 0.327 ± 0.042, with statistical significance. Conclusions: This study successfully transfected β-globin gene and α-globin gene siRNA into K562 cell. After lentiviral transfection, the levels of β-globin gene RNA and protein were significantly higher than the control group.

Keywords: β-thalassemia, β-globin, α-globin, K562 cell, lentiviral vector, gene therapy

Introduction

Beta- (β-) thalassemia is a kind of hereditary hemolytic anemia disease which is seriously harmful to human health. The disease is caused by reduce or cease of the synthesis of β-globin peptide induced by point mutation or deletion of β-globin gene and their regulatory sequence [1-3]. In recent years, a new virus vector, Human Immunodeficiency Virus (HIV) lentivirus vector, has been successfully used to the gene therapy of β-thalassemia [4-7]. Especially for the β-globin deficiency caused by the splice switching and termination codon through the defective β-globin gene, this type of thalassemia can make lentivirus mediate RNA interference and lead the recovery of the abnormal expression of antisense sequences of splice sites of mRNA precursors of intron 1 (such as IVS-I-110, IVS1-6, and IVS1-5) or aberrant splicing of intron 2 (such as IVS-II-654 and IVS-II-745), which can restore the correct splicing sequence and eventually restore the synthesis of hemoglobin [8-12].

However, studies about translation defective thalassemia are rare. Because this type of thalassemia had large deletion fragments, it cannot be restored to normal base expression and structure of polypeptide chain through RNA
Lentivirus vectors α-globin interference β-thalassemia for translating repair and interference technology to improve hemoglobin expression. Therefore, we can only adjust the α/β globin proportion though the introduction of normal human β-globin gene supplement or reduce of α-globin gene expression. But the efficiency of α-globin gene interference, the effectiveness of β-globin gene and its regulatory fragment vector and the long term stability of α/β ratio control and coordination have not been effectively verified.

In this study, we focused on the most common type of thalassemia, β-thalassemia mutations in codons 41-42 translation defection, in Guangxi Province of China, and designed two lentiviral vectors, which means when the lentivirus mediated full-length human β-globin gene, we introduced lentivirus α-globin gene interference fragment to adjust the α/β ratio by increasing the β-globin and decreasing the α-globin. This study will provide more effective evidence and strategy for the gene therapy of β-thalassemia in Guangxi Province and even China. Moreover, the design and construction of various gene vectors can establish foundation for gene knockout mouse model construction and in vivo gene therapy of CD41-42 type of thalassemia.

**Materials and methods**

**Materials**

We obtained peripheral blood sample from a case with normal globin sequence that had no thalassemia through gene detection in the Guangxi Key Laboratory of Thalassemia. Main reagents included QIAGEN plasmid Mini Kit, QIAquick Gel Extraction Kit (German QIAGEN Company), Lipofectinamine 2000 (Shanghai Invitrogen Company), SYBR Master Mixture, and Quantitative PCR Kit (Swiss Roche). Main vectors and antibodies included lentivirus vectors pLVEF1a/GFP+Puro (Shanghai Genechem Co., Ltd.), recombinant shuttle plasmid and packaging plasmid PGCSIL-017 vector (Shanghai Genechem Co., Ltd.), Mouse monoclonal antibody against human β-globin (American Abnova Corporation), Mouse monoclonal antibody against human α-globin (American Abnova Corporation), and goat anti-mouse IgG secondary antibody (Beijing Boisynthesis Company). Major instruments included Time PCR Real instrument TP800, fluorescence microscope Ts100/3.3RTV (TAKARA Company), Japan Nikon Eclipse/OLYMPUS, fluorescence quantitative PCR instrument 7500 (American ABI Corporation), and gel imaging analysis system (American BIO-RAD Corporation).

**Experimental methods**

We extracted the β-globin gene sequence in normal human and constructed the lentiviral vector (LV-β-globin) containing the full-length human β-globin gene. The detailed methods have been published elsewhere. We constructed the RNA interference vector (red fluorescence) for α-globin gene. We designed four best siRNA interference sequences by using the RNA interference sequence design principle according to the α-globin gene (HbA2) sequence (Table S1).

We made clone preparation and PCR identification of positive clones. PCR identification system and PCR program were as follows: Primer (+): CCATGATTCCCTCATATTGC; Primer (-): GTAATACGGTT ATCCACGCG. Reaction system was as follows: Template 1 µl, 10× Pfu Buffer (+Mg²⁺) 2 µl, dNTP (2.5 mM) 0.8 µl, Primer (+)(-) each 0.4 µl, Taq polymerase 0.2 µl, and ddH₂O 20 µl. Reaction conditions were as follows: 94°C for 30 s with 1 cycle; 94°C for 30 s, 60°C for 30 s, 72°C for 30 s with a total of 30 cycles, and 72°C for 6 min with 1 cycle.

We transfected 293T cells using the lentiviral vectors with correct sequence for packaging. Target cell infection test was conducted by adding the lentiviral particles according to the designed groups. The expression of RFP was observed under fluorescence microscope after infection, and the titer was measured by fluorescence method. According to the cell MOI value (low MOI value 20, and high MOI value 40), we added the appropriate amount of virus. The expression of the reporter gene RFP was observed after 3 days of infection. The reference gene and target gene primers were designed and synthetized by Shanghai Jikai Company.

Reaction system was allocated according to the following proportion: 10 µl of SYBR premix ex taq, 0.5 µl of upstream primer (2.5 μM), 0.5 µl of downstream primer (2.5 μM), 1 µl of cDNA, and 8 µl of RNase-Free H₂O. PCR Real-Time is divided into two steps and the melting curve was produced. The relative quantitative detec-
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real-time PCR was conducted to verify the expression of the globin gene. The primer design is showed in Table S2.

We conducted Western-blotting to further verify the expression of α-globin and β-globin. The protein sample preparation was conducted using protein quantification method (BCA method). Appropriate amount of working fluid was prepared according to the instructions. Protein electrophoresis and immune reaction were also conducted. The grey value of protein was analyzed by Quantity One v462 software (grey value of target protein = OD value of target protein/corresponding reference OD value). Three independent Western blotting OD values were conducted and the mean and standard deviation were calculated.

Statistical methods

Statistical analyses were conducted using SPSS 19.0 software. The variables in each group were described as \(\bar{x} \pm S\), and univariate analyses between two groups were conducted by t-test. \(P\) value of <0.05 was considered as statistically significant.

Results

Package results of LV-β-globin lentiviral vector

Lentiviral packaging system was used to package 293T cells in the logarithmic growth phase, and the lentiviral was packaged successfully. The LV-β-globin lentiviral titer reached 1×10^8 determined by fluorescence method. K562 cells were transfected using lentivirus LV-β-globin 1:10-1:100 with a titer of 1×10^8 TU/ml. Fluorescence expression was intense when infected with MOI value of 20. The stable transfection cell lines were screened by flow cytometry. The infection rate was 94.8%, while the blank group and the negative control group showed no fluorescence. The results of cell infection are shown in Figure 1.

Detection results of RNA interference lentivirus vector for α-globin gene

Positive recombinant PCR bands results indicated that the size of positive clone PCR fragment connected to the vshRNA fragment was 342 bp and the size of the empty vector clone PCR fragment without connection to the vshRNA fragment was 307 bp. Titer of virus packaged cell 293T determined by fluorescence method: LV-HbA2-RNAi(1) 3×10^8; LV-HbA2-RNAi(2) 2×10^8; LV-HbA2-RNAi(3) 2×10^8; LV-HbA2-RNAi(4) 3×10^8. The results of cell infection are shown in Figure 2, and the results of real-time PCR are shown in Tables 1 and 2.

The quantitative PCR results showed that in K562 cells, compared with the negative control group, HbA2 gene knock reduction efficiency of KD2 (LV-HbA2-RNAi/2) with high MOI value reached 76.5% \((P<0.05)\), and KD2 (LV-HbA2-RNAi/2) was considered as an effective target.

Figure 1. The K562 cells infected by human β-globin gene lentivirus. NC = negative control group; CON = blank group; OE = gene addition group; 100× and 200× represent a magnification of 100 times and 200 times; b and g represent bright field and fluorescence field of vision.
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K562 cells of human β-globin gene transfected by LV-HbA2-RNAi lentivirus

Twenty-four hours after transfection, red fluorescence expression was visible under fluorescence microscope; after 72 hours, the cells grew in good condition and the fluorescence expression was strong. The infection efficiency is about 91.3% by green fluorescence and red fluorescence when infected by lentivirus with

Figure 2. Cell infection of lentivirus RNAi. -1 and -2 represent low MOI and high MOI; 100× and 200× represent a magnification of 100 times and 200 times; b and g represent bright field and fluorescence field of vision (r: red fluorescence field).
MOI value of 40 and titer of $1 \times 10^8$ TU/ml, and the cells expressed green fluorescence observed using green fluorescence microscopy. There was no expression of red fluorescence in the blank group and only the expression of green fluorescence. The results are shown in Figure 3.

**The results of globin gene expression verified by real time PCR**

β-globin gene was expressed in the stable transfected K562 cells, and amplified specific bands of 227 bp were obtained. However, it was not expressed in the stable transfected K562 cells in the control group (Figure 4). Relative quantitative detection of HBB mRNA indicated that the relative expression of the K562 cell line with stable gene expression showed significant difference ($P<0.05$), and the expression level of HBB was $4.080 \pm 0.078$, which was 4.08 times of blank group (Table S3). Relative quantitative detection of HbA2 mRNA indicated that the relative expression of the K562 cell line after stable interference showed significant difference between the groups ($P<0.05$), the HbA2 expression level was $0.274 \pm 0.023$, and the interference efficiency of blank group was 72.3% (Table S4).

**Expression results of α- and β-globin verified by Western-blotting**

The expression of β-globin gene protein synthesis in the experimental group was significantly higher than that of the blank group and negative control group after introducing LV-β-globin lentivirus in K562 cells. Western-blotting results showed that grey value of β-globin increased by 3.6 times, which was significantly higher than that of the blank group and negative control group ($F = 32.752, P<0.001$). There was no statistical difference between the blank group and the negative control group ($t = 0.12, P>0.05$) (Figure 5; Table S5).

The synthesis of α-globin was significantly lower than that of negative control group after introducing α-globin-RNAi in the K562 cells. Western-blotting results showed that grey value of α-globin in the α-globin-RNAi group decreased by 68.3%, which was significantly lower than that of the blank group and negative control group ($F = 14.752, P = 0.016$). There was no statistical difference between the blank group and the negative control group ($t = 0.032, P>0.05$) (Figure 6; Table S6).

**Discussion**

In this study, we successfully transfected K562 cells with the constructed full-length human LV-β-globin and α-globin-RNAi. We also confirmed that the expression of β-globin increased and the expression of α-globin decreased. Our study confirmed the gene therapy concept and laid foundation for therapy of β-thalassemia.

β-thalassemia is a hereditary hemolytic disease which is seriously harmful to human health. Epidemiological investigation showed that the carrier rate of thalassemia and other abnormal hemoglobin was 24.51% in Guangxi Province, with carrier rate of β-thalassemia 6.43%. The molecular pathogenesis of β-thalassemia is that the synthesis of β-globin is reduced or creased due to the β-globin gene mutation or deletion, which lead to excessive synthesis of α-globin. The excessive α-globin deposits on the cell membrane, causing direct damage of erythrocyte membrane. Further-
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more, the excessive α-globin chain deposits together to form inclusion bodies, and the lifespan of red blood cells are negatively related to the number of their inclusion body. When the inclusion bodies are combined with red cell membrane, the deformation ability decreases, and the red blood cells are easily damaged by the spleen. When α-globin chain deposits on the red blood cell membrane, the progenitor cells are destroyed by the hemolytic mechanism. In addition, the number of apoptosis in patients with severe β-thalassemia is 15 times or even higher than that in normal people. Animal experiments show that the α/β chain ratio determines the proportion of apoptosis and ineffective hematopoiesis in the mouse model of thalassemia, which suggests that the apoptosis of erythrocyte progenitor cells is related to the α-globin chain deposition.

In recent years, lentiviral vector has been successfully applied to the gene therapy of β-thalassemia. Especially splice switching and stop codon readthrough defects of the β-globin gene expression leads to a lack of β-globin. Lentivirus mediated RNA interference technology make the precursor mRNA in aberrant splicing of the antisense sequence of intron 1 (such as IVS-I-110, IVS-I-6, and IVS-I-5) or intron 2 (such as IVS-II-654 and IVS-II-745) restore successfully, and eventually restore hemoglobin synthesis [13-17]. Studies have shown that the effective rate of interference at the cellular level for β-thalassemia of shear switch and stop codon readthrough defects can achieve about 70% recover [18], and the symptoms of anemia in mice obtained apparent ease [19]. In addition, shear switching of oligonucleotides is also an effective method to restore the expression of β-globin in abnormal

Figure 3. The K562 cells infected by siRNA lentivirus. 100× and 200× represent a magnification of 100 times and 200 times; b and g represent bright field and fluorescence field of vision (g: green fluorescence field, r: red fluorescence field).

Figure 4. PCR electrophoregrams of α-globin gene and β-globin gene in K562 cells. M: DL 600 DNA maker; Lane 1: GAPDH and β-globin gene; Lane 2: GAPDH; Lane 3: GAPDH, α-globin gene.
Many researchers try to treat β-thalassemia by decreasing the proportion of α-globin/non α-globin chain synthesis [22], which means they try to reduce the α-globin chain synthesis and increase β-globin chain synthesis to improve symptoms in patients with β-thalassemia. For example, hydroxyurea therapy can induce fetal hemoglobin (HbF) generation and increase non β-globin chain proportion to compensate β-globin chain synthesis problems, and improve blood and clinical symptoms [23].

Gene replacement therapy is a method that introduces normal β-globin gene into the body to replace the abnormal β-globin gene in patients to improve the expression of normal β-globin and balance proportion of α-globin/non α-globin chain synthesis [24]. Chinese researchers used antisense oligonucleotides which could effectively inhibit the expression of human α-globin gene to improve the α/β-globin gene imbalance [25].

Xie et al. [8] designed α-globin siRNA vector (Mouse-α1 and Mouse-α2) specific for mouse and α-globin siRNA vector (H-α1, H-α2 and H-α3) specific for human and transfected with siRNA to the mice and K562 cells respectively [26]. The results showed that the expression of α-globin mRNA and protein in mice and K562 cells was effectively inhibited, but the interference inhibition rate of the three siRNAs was all lower than 50%. In the current study, the inhibition rates were 76.5% and 72.3%, respectively, which were significantly higher than those of previous studies. Moreover, the use of novel lentiviral vector can infect the whole cell line no matter in cells or animals, and the fragments and gene silencing function will be more stable and continuously expressed in vitro and in vivo tests, which is more benefit to use in the gene therapy with better safety than the previous studies. In this study, we effectively inhibited the synthesis and expression of mRNA and globin, which laid the foundation for reducing α-globin deposition and balancing the proportion of α/β-globin in patients with β-thalassemia.

K562 cells are human erythroleukemia cells, which mainly synthesize hemoglobin. That means this cell mainly expresses human α-andy-globin chain and is a good cell model for research on human blood diseases. Because this cell line expresses α- and γ-globin chain and almost don’t express human β-globin, thus, it is a very good tool to study the transition mechanism of γ-globin to α-globin in the development process. Zoueva et al. [27] successfully enhanced the amount of β-globin mRNA in the K562 cells by reducing β-globin inhibitory factor. Some researchers conducted β-thalassemia treatment study by activating the expression of β-globin, [28] which was a very effective cell model for reducing the expression of α-globin and reducing α-globin deposition in patients with β-thalassemia. In this study, according to the characteristics of K562 cells and specificity of the design and construction in human α2-globin expression, we focused on siRNA expression vector of human α2-globin chain and transfected K562 cell line to observe the inhibiting effect of α2-globin chain expression. At the level of mRNA and protein expression, design and construction of RNAi vector can effectively inhibit the expression of α2-globin chain and expression of α-globin in K562 cells, indicating that it is feasible to treat β-thalassemia by inhibiting α-globin expression and balancing the proportion of α/β-globin.

In conclusion, we successfully constructed and packaged lentiviral that contained normal human β-globin gene. We further stably transfected K562 cells to make the expression of β-globin normal and created gene based mate-
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terials and conditions. We also successfully constructed the lentiviral vector of human α-globin RNAi and provided stable transfection of lentiviral vector for RNA interference in vivo on gene therapy of β-thalassemia. Moreover, we successfully constructed the human β-globin gene lentiviral and α-globin gene siRNA lentivirus and stably transfected into K562 cells. The level of β-globin gene mRNA was 4.08 times higher than that of the control group and the level of protein expression was 3.6 times higher than that of the control group. In the final stable transfected cells, the mRNA knock down efficiency was 72.3%, and the protein knock down efficiency was 68.3%. This study provided more effective evidence and strategy for the gene therapy of β-thalassemia by adjusting the α-/β-globin chain ratio and laid foundation for gene knockout mouse model construction and in vivo gene therapy of thalassemia in the next step.

Acknowledgements

Guangxi Key Laboratory Project (15-140-11)
Guangxi Medical and Health Key Research Projects 2012063; The Small Talent Heights of Guangxi University - Project for Team to Assisted Reproductive Technology and Prevention and Control Innovation in Genetic Disease; The Guangxi medical scientific research center of open fund project KFJJ2010-15.

Disclosure of conflict of interest

None.

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References

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Table S1. SiRNA sequence for HbA2

<table>
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<th>NO.</th>
<th>Target sequence information</th>
<th>GC content (%)</th>
<th>Start Pos.</th>
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<tr>
<td>HbA2-RNAi(1)</td>
<td>TGGCTTCTGTGACGACCGT</td>
<td>57.89%</td>
<td>453</td>
</tr>
<tr>
<td>HbA2-RNAi(2)</td>
<td>AGCTCTAAGCCACTGCTT</td>
<td>57.89%</td>
<td>363</td>
</tr>
<tr>
<td>HbA2-RNAi(3)</td>
<td>ACGGCAAGAAGCTGGGCGGA</td>
<td>63.16%</td>
<td>240</td>
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<tr>
<td>HbA2-RNAi(4)</td>
<td>AGGTAAAGCCACCGCCA</td>
<td>57.89%</td>
<td>228</td>
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Table S2. Primer sequences of HbA2 gene and reference gene

<table>
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<tr>
<th>Reference gene</th>
<th>Upstream primer sequences</th>
<th>Downstream primer sequences</th>
<th>Amplified fragment size (bp)</th>
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<tr>
<td>GAPDH</td>
<td>TGACTTCAACAGGACACCCA</td>
<td>CACCCTGTTCTGCTAGCCCAA</td>
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<td>Target gene</td>
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<tr>
<td>HbA2</td>
<td>CACGCTGCGGAGTATGGT</td>
<td>GGTCGAAGTGCGGGAAGT</td>
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Table S3. Description of sample grouping in testing HBB gene by Real-time PCR (± S)

<table>
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<th>Sample</th>
<th>Average ± STDEV (2^ΔΔCt)</th>
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<tr>
<td>CON</td>
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<tr>
<td>NC</td>
<td>0.982 ± 0.015</td>
</tr>
<tr>
<td>OD</td>
<td>4.080 ± 0.078</td>
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<tr>
<td>P (CON-OD)</td>
<td>0.0011</td>
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<tr>
<td>P (NC-OD)</td>
<td>0.0020</td>
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Table S4. Description of sample grouping in testing HbA2 gene by Real-time PCR (± S)

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<td>CON-2</td>
<td>0.963 ± 0.078</td>
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<tr>
<td>NC-2</td>
<td>1.004 ± 0.108</td>
</tr>
<tr>
<td>KD</td>
<td>0.274 ± 0.023</td>
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<tr>
<td>P (CON-KD)</td>
<td>0.0041</td>
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<tr>
<td>P (NC-KD)</td>
<td>0.0073</td>
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Table S5. Detection of β-globin protein grey value of by Western blotting (± S)

<table>
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<th>CON</th>
<th>NC</th>
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<tr>
<td>Protein grey value</td>
<td>0.292 ± 0.017</td>
<td>0.322 ± 0.066</td>
<td>1.063 ± 0.082*</td>
</tr>
</tbody>
</table>

*Compared with CON and NC, P<0.0001.

Table S6. Detection of α-globin protein grey value of by Western blotting (± S)

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<th>CON</th>
<th>NC</th>
<th>KD</th>
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<td>Protein grey value</td>
<td>0.980 ± 0.027</td>
<td>1.010 ± 0.056</td>
<td>0.327 ± 0.042*</td>
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*Compared with CON and NC, P<0.05.