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ORIGINAL ARTICLE

Lyophilization is suitable for storage and shipment of fresh tissue samples without altering RNA and protein levels stored at room temperature

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Abstract Lyophilization has been widely used for preservation, such as in food industry, pharmacy, biotechnology and tissues engineering, etc. However, there is no report on whether it could affect stability of RNA and protein levels in biological tissue samples. Herein we show that lyophilization can be used for storage of biological tissue samples without loss of bioactivities even stored at room temperature for 7–14 days. To address this issue, C57BL mouse tissues were prepared and dried by lyophilization and a baking method, respectively, followed by examination of morphological structure and total proteins by SDS-PAGE as well as gelatin zymography. Subsequently, the stability of RNAs and proteins, which were lyophilized and stored at room temperature (23°C) for 14 days was further examined by RT-PCR, SDS-PAGE and western blot. Results demonstrated that lyophilization did not alter total protein activities of various tissues, including enzyme activities, immunoreactivities and phosphorylation, and did not affect several RNAs in lyophilized tissues. Taken together, lyophilization may represent a valuable approach for preservation and long-distance shipment of biological samples, particularly for the international exchange of biological samples without altering their bioactivities.

Keywords Lyophilization · Tissue storage · Tissue transferring · RNA · Protein · Bioactivity

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Introduction

Fresh or frozen tissue samples are widely used in basic medicine and biology studies. Preservation of them keeping their bioactivities represents an important problem, particularly for preservation and long-distance shipment of biological samples for international exchange. Previous studies have shown that several techniques could be used for tissue sample long-term preservation, such as liquid nitrogen (Yogev et al. 2010) and dry ice (Woods et al. 2010). However, these methods are unsuitable for storage and/or international exchange because of the potential safety, costs and inconvenience for operation.

Lyophilization was initially developed in World War II with the need of serum sample storage and has been becoming a routine molecular biological method in recent years (Devireddy and Thirumala 2011). Lyophilization has been widely used for freeze-drying and storage of various biological samples in food industry, pharmacy, biotechnology and tissues engineering, such as plant seeds (Schafer et al. 2008), bacteria (Bolla et al. 2010), yeast cells

(Miyamoto-Shinohara et al. 2010), bovine pericardium (Leirner et al. 2009; Santibanez-Salgado et al. 2010), liposomes (Stark et al. 2010), mouse spermatozoa (Kawase et al. 2009; Watanabe et al. 2009) and rat kidney (Nygren et al. 2005), to name a few. This favorable technology could effectively keep bioactivities of samples (Adams 2003; Chen et al. 2010; Matejtschuk 2007). However, whether this treatment could influence stability of RNAs and proteins in biological tissue samples, even when samples are kept at ambient temperature, has not been reported before. The current study demonstrates that lyophilization is an ideal approach because it is not altering protein activity of various tissues, such as enzyme activities, immunoreactivities, phosphorylation, and preserves nucleic acid integrity in lyophilized tissues.

Results

Lyophilization did not change the morphological appearance, i.e., shape and total proteins in mouse lyophilized tissues

To elucidate whether lyophilization changes morphological structure and protein activity of biological tissue samples, C57BL mice tissues were prepared and photographed (Suppl Fig. 1). The overall morphological appearance of lyophilized tissues showed no differences between shapes compared with controls, but they were seriously shrunk in the baking preparation tissues. Furthermore, total proteins of C57BL mice tissues were examined by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). There were no significant differences for the total proteins between the lyophilized tissues and control, but not for the baking tissues because these were completely degraded by the high temperature of 80°C.

Activities of matrix metalloproteinases (MMPs) were also examined by gelatin zymographical analysis in lyophilized tissues, which were stored at room temperature of 23°C for 14 days (Fig. 2). Enzyme activities of MMP-2 and MMP-9 were not changed in lyophilized heart, liver, spleen and stomach, following storage at room temperature for 2 weeks as compared to controls. In the lyophilized kidney, MMPs were showing reduced activity on day 14 and in brain from day 7.

These data suggest that lyophilization is a valuable technology not only for stabilizing the overall morphological appearance of the biological tissues but also for maintaining the activity of the total proteins.

Lyophilization did not change RNA of lyophilized mouse brains even by storage at room temperature for 2 weeks

To further analyze potential changes of RNA in the lyophilized tissues following storage (1, 3, 7, and 14 days)

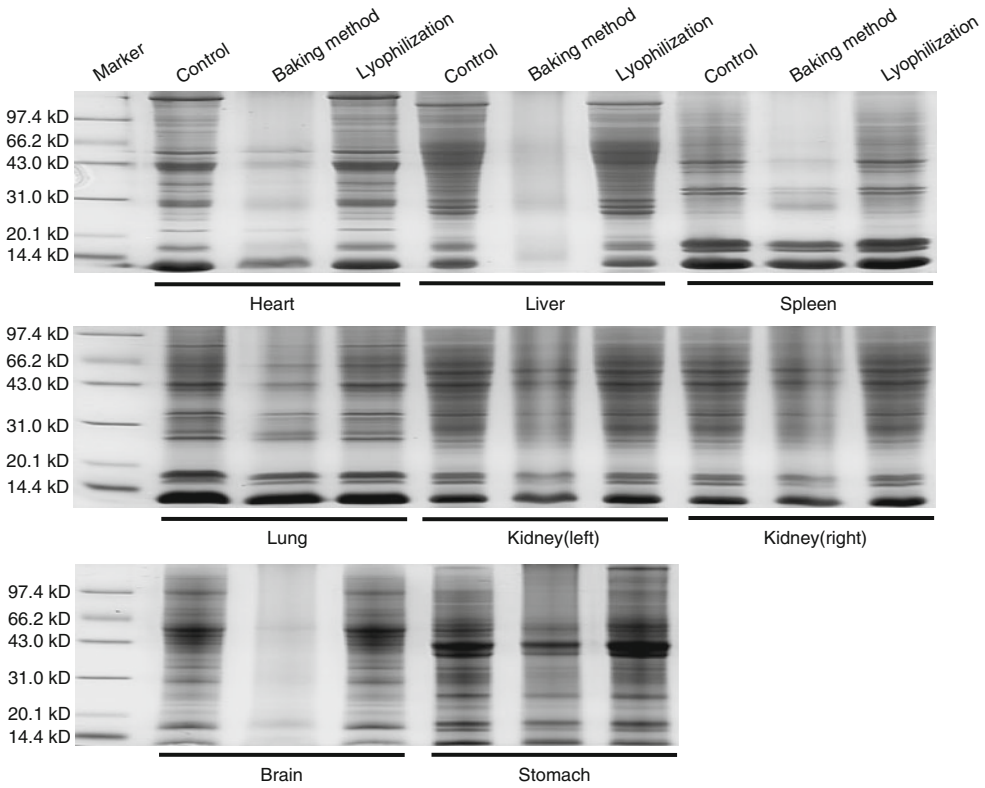

Fig. 1 SDS-PAGE identified the change of the total protein patterns in the C57BL mice baked and lyophilized tissues. A remarkable difference of the electrophoretic pattern was observed in baked samples

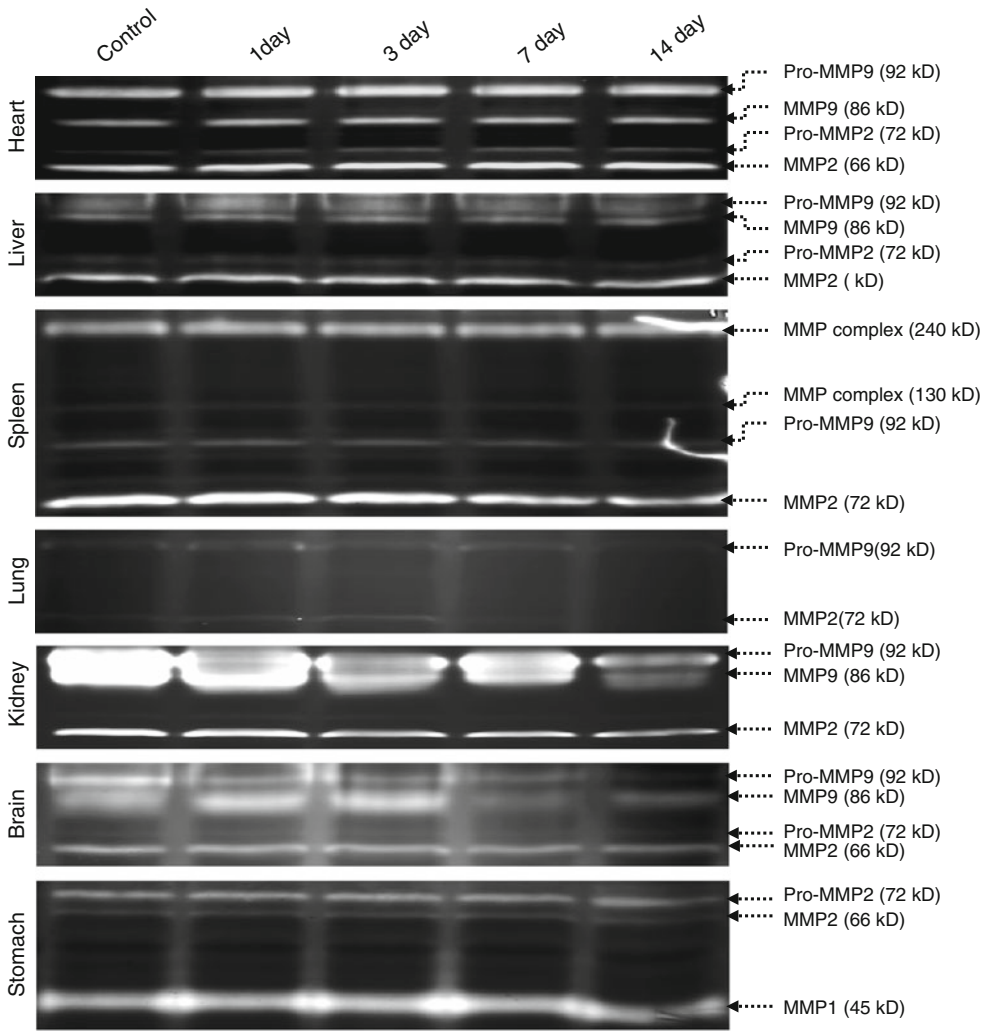

Fig. 2 Gelatin zymography demonstrating the activity of MMP2 and MMP9 in the C57BL mice lyophilized tissues after storage at room temperature for up to 14 days. The enzyme activities of MMP2 and MMP9 are provided

at room temperature, brain tissues were examined: total RNA was prepared and randomly selected three RNAs (GAPDH, VEGF and Cox-2) were amplified by RT-PCR subsequently (Fig. 3). There was no significant difference between RNAs from 1 to 14 days storage at room temperature. Therefore, lyophilization did not alter stability of mRNA in the lyophilized tissues.

Lyophilization did not alter protein immunoreactivity and phosphorylation in lyophilized mouse brains even by storage at room temperature for 2 weeks

To elucidate on whether the proteins' immunoreactivity and phosphorylation in lyophilized mouse brains could be altered by storage at room temperature, C57BL mice brain tissues were also prepared by lyophilization. The total proteins were extracted and identified by SDS-PAGE (Fig. 4a). Subsequently, the randomly selected proteins, Notch-1, pERK1/ERK2 and GAPDH, were analyzed by western blot (Fig. 4b, c). Lyophilization followed by storage at room temperature did not affect the total protein amounts, as well as the proteins' immunoreactivity and phosphorylation. These data indicate that lyophilization is a valuable and safe technology for storage of biological tissue samples.

Discussion

While lyophilization of individual proteins is a standard method of protein preservation, the use of lyophilization for preserving integrity of macromolecules in tissues has not been systematically studied yet, to the best of our knowledge.

Drying of tissues by baking did not point to any useful further applications; freeze-drying (lyophilization), however, kept morphological appearance as evaluated by inspection and enabled further use for SDS-gel electrophoretical studies as proposed based upon comparison of patterns on SDS-PAGE of different organs. This finding

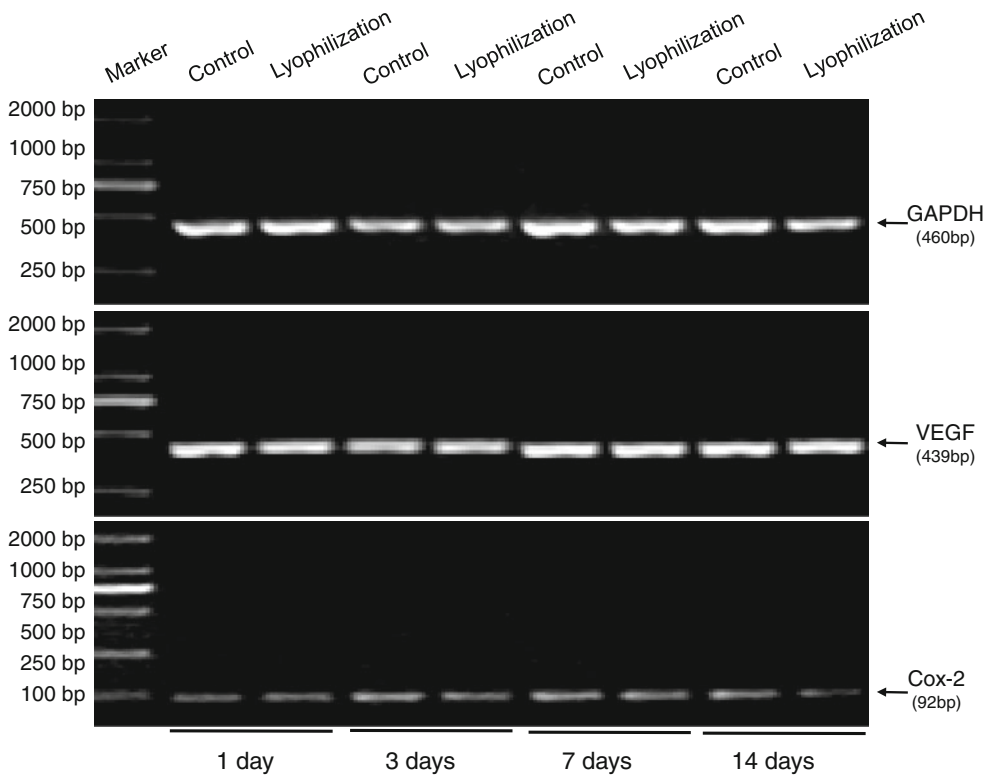

Fig. 3 RT-PCR detected stability of RNAs in lyophilized tissues and the agarose electrophoretic pattern is displayed: 2% agarose-gel electrophoretic analysis of the expression of GAPDH, VEGF and Cox-2 by RT-PCR. The corresponding histogram showing the expression levels of GAPDH, VEGF and Cox-2 is given in supplemental Fig. 2

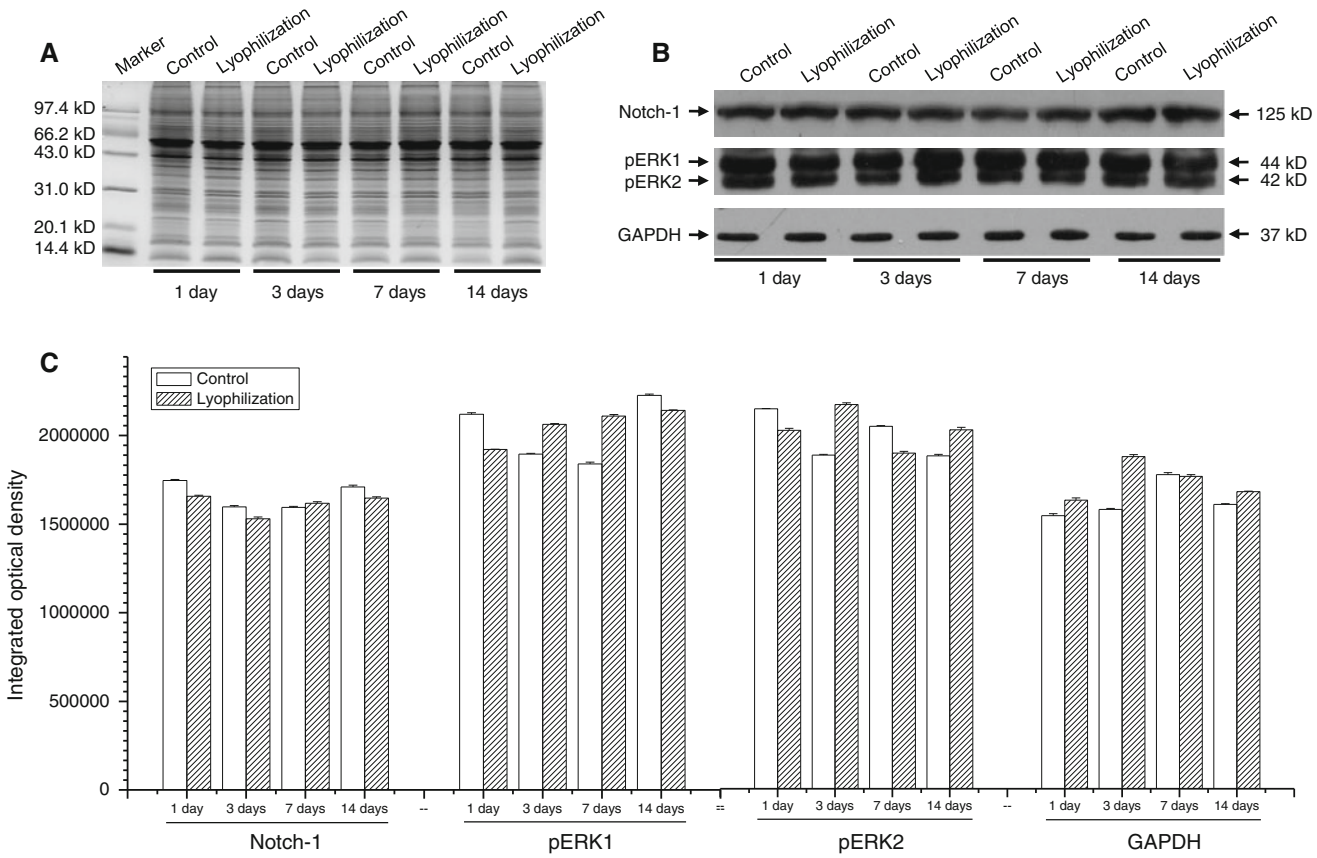

Fig. 4 SDS-PAGE and western blots revealed comparable electrophoretic protein patterns in lyophilized brains. **a** SDS-PAGE analysis of the total brain proteins. **b** Western blot analysis of Notch-1, pERK1/ERK2 and GAPDH. **c** Histogram showing comparable levels of Notch-1, pERK1/ERK2 and GAPDH

indicates that electrophoretic studies on shipped lyophilized samples shows no changes in electrophoretic mobility or pattern per se, although it remains open if two-dimensional gels on lyophilized samples would reveal electrophoretic changes, e.g., shifts.

SDS-PAGEs of total proteins as well as of individual proteins were indicating patterns consistent with preservation of quality and quantity. Indeed, protein stability remains a major problem in science and technology (Deutscher 2009). Phosphorylation of phospho-ERKs was stable over the whole period of 14 days as observed by immunoblotting. This information is important because lability of phosphorylation is a major issue in proteomics (Mandell 2003; Sun and Julian 2011). Work on zymography has shown that in organ- and time-dependent way, proteolytic activities can be preserved for up to 14 days.

The current study also demonstrates that polymerase chain reaction (PCR) of lyophilized samples resulted in RNA patterns comparable to controls as given by optical density. Minor changes, qualitative or quantitative in nature, however, may not have been observed using the technique. This first preliminary report shows that lyophilization may show potential for preservation and shipment of valuable samples, which is important for scientific cooperation worldwide. A series of questions, however, remain open, including the question of conformational changes or other post-translational modifications. In addition, one may not be able to generalise preservation for all proteins because protein stability may vary with structure and physicochemical properties of the individual proteins.

However, this first approach might challenge further studies on freeze-drying as a potentially valuable approach in particular, as shipping costs for using dry ice or liquid nitrogen are soaring.

Materials and methods

C57BL mice tissues preparation and treatment

Twenty-four C57BL mice (18–20 g) were provided by Vital River Inc. (Beijing, China) and randomly divided into two groups, baking method (80°C, 6 h) and lyophilization (−50°C, 12 h, atmospheric pressure 100 Pa) (Table 1). Mice in the baking preparation group were dissected to obtain the heart, liver, spleen, lung, kidney, brain, and stomach, and quickly removed and washed by normal saline. Subsequently, these tissues were directly dried by drying oven (9240A, Shanghai Cany Precision Instrument Co., LTD, China) for 12 h, and then took photographs and extracting total proteins for SDS-PAGE examination. Mice in the lyophilization group were also dissected to obtain the heart, liver, spleen, lung, kidney, brain and stomach, and quickly removed and washed by normal saline. Subsequently, these tissues were pre-frozen in a −80°C freezer for 12 h and lyophilized by a −50°C vacuum freeze-drying instrument (FD-1 A-50, Beijing Boyikang Instrument Co., LTD, China) for 24 h, and then storage at room temperature (23°C) for 1, 3, 7, and 14 days (Table 1). Total RNA and total protein of mouse brains were extracted and identified by RT-PCR, SDS-PAGE and western blot for all the animals. Each group contained one control animal and was treated as the experimental group followed by storage at −80°C for further use.

Imaging and total protein preparation

Mouse tissues for the mice of both the baking and lyophilization preparation group were photographed for comparison of morphological appearance. Subsequently, total protein of the animal tissues were extracted by cell lysis buffer (including 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM sodium orthovanadate, 1 mM sodium fluoride, 1 mM EDTA and 1 mM protease inhibitor) and further identified by SDS-PAGE and western blot.

Gelatin zymography of lyophilized mouse tissues

The total proteins of lyophilized tissues, stored at room temperature for 1, 3, 7 and 14 days, were extracted and gelatin zymography according to a previous report was performed (Hawkes et al. 2010).

RT-PCR assay

Total RNA of C57BL mouse brains of the lyophilization preparation group was extracted by Total RNA kits II

Table 1 Experimental groups

Tissue preparation	Baking method ($n = 4$)	Lyophilization ($n = 20$)				
Storage time at room temperature	0 days	0 day	1 day	3 days	7 days	14 days
Number of mice used for control	1	1	1	1	1	1
Number of mice used for tissue preparation	3	3	3	3	3	3

(Omega, Japan) according to the manufacturers' instructions, and then reverse-transcribed by the Reverse Transcription System (Sigma, USA). Subsequently, the three randomly selected RNAs, GAPDH (460 bp), VEGF (439 bp) and Cox-2 (92 bp) were amplified by PCR using the following primers: GAPDH-forward: 5′-ACCACAGT CCATGCCATCAC-3′, GAPDH-reverse: 5′-TCCACCAC CCTGTTGCTGTA-3′. VEGF-forward: 5′-TGTGAGCCT TGTTCAGAGCGGAGAA-3′, VEGF-reverse: 5′-AATCT TCCGGGCTTGGCGATTTAGC-3′. Cox-2-forward: 5′-ATC GCTGTACAAGCAGTGGCAAAGG-3′, Cox-2-reverse: 5′-AGGGAGAAGCGTTTGCGGTACTCAT-3′. PCR products were identified on 2% agarose-gel electrophoresis followed by image analysis using integrated optical density (IOD; ImageJ software; http://rsb.info.nih.gov/ij/, NIH), and then plotted by Origin 8.1 software. All experiments were repeated at least three times.

SDS-PAGE and western blot assay

Forty microgram of total proteins from C57BL mice tissues in both, the baking and lyophilization preparation group, were fractionated by electrophoresis with 12.5% SDS–polyacrylamide gels and stained by Coomassie Brilliant Blue R-250 at room temperature for 2 h.

Fractionated gels were then transferred to a polyvinylidene difluoride (PVDF) membrane (GE, USA) following the manufacturer's instructions. The membrane was probed with rabbit-derived anti-Notch-1 antibody (1:100 in TBST, Abcam), mouse-derived anti-pERK1/ERK2 antibody (1:1,000 in TBST, Sigma), and mouse-derived anti-GAPDH first antibody (1:500 in TBST, Beijing, Zhong-Shan Biotechnology) for 1.5 h at room temperature and developed with the HRP-conjugated goat anti-mouse or anti-rabbit secondary antibody (1:5,000 in TBST, Beijing Zhong-Shan Biotechnology) by incubation for 1 h at room temperature. Chemiluminescence substrate luminal reagent (GE, USA) was used to detect immunolabeled bands by exposure to X-ray films. Protein bands were also analyzed using the above-mentioned ImageJ software. All experiments were repeated at least three times.

Statistics

All the above data were analyzed by the SPSS software (version 13.0, SPSS, USA) using Student's t test with significant differences defined at $P < 0.05$.

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Conflict of interest None.

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