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The influence of storage parameters on measurement of survival motor neuron (SMN) protein levels: Implications for pre-clinical studies and clinical trials for spinal muscular atrophy

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Abstract

Spinal muscular atrophy (SMA) is caused by low levels of survival motor neuron (SMN) protein. A growing number of potential therapeutic strategies for SMA are entering pre-clinical and clinical testing, including gene therapy and antisense oligonucleotide-based approaches. For many such studies SMN protein levels are used as one major readout of treatment efficacy, often necessitating comparisons between samples obtained at different times and/or using different protocols. Whether differences in tissue sampling strategies or storage parameters have an influence on measurable SMN levels remains to be determined. We assessed murine SMN protein immunoreactivity over time and under differing tissue storage conditions. SMN protein levels, measured using sensitive quantitative fluorescent western blotting, declined rapidly over a period of several days following sample collection, especially when protein was extracted immediately and stored at −20 °C. Storage of samples at lower temperatures (−80 °C), and as intact tissue, led to significantly better preservation of SMN immunoreactivity. However, considerable deterioration in measurable SMN levels occurred, even under optimal storage conditions. These issues need to be taken into consideration when designing and interpreting pre-clinical and clinical SMA studies where SMN protein levels are being measured.

Keywords: Spinal muscular atrophy; SMN; Western blot; Clinical trials; Mouse; Biomarker

1. Introduction

Proximal spinal muscular atrophy (SMA) is a leading genetic cause of infant mortality [1], resulting from homozygous deletion or mutation of the survival motor neuron 1 (*SMN1*) gene [2]. Humans have two *SMN* genes (*SMN1* and *SMN2*), but the *SMN2* gene produces considerably less full-length SMN protein meaning that it cannot fully compensate for loss of the *SMN1* gene [3,4]. The major pathological characteristic of SMA is loss of lower alpha motor neurons from the ventral horn of the spinal cord, resulting in progressive muscle denervation and atrophy, particularly of the proximal muscles [5,6].

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Several novel therapeutic approaches for SMA are showing promise in pre-clinical studies [6], including; gene therapy [7–10], anti-sense oligonucleotides [11–15], histone deacetylase (HDAC) inhibitors [16–19], and beta-catenin inhibitors [20]. Indeed, several of these approaches are entering clinical trials in patients. In the absence of validated, robust biomarkers [21–25] SMN levels are often used as a major read-out to evaluate therapeutic efficacy. However, proteins can be unstable when outside their native environment. If this were applicable to SMN, it is possible that patient or animal samples obtained at different times and/or processed and stored in different ways (as may be likely in a multi-centre clinical trial) could show changes in levels of SMN protein as a consequence of their handling and storage rather than as a result of actual differences in SMN protein levels.

2. Materials and methods

2.1. Animal models

Young adult (1 month old) wild-type CD1 mice were obtained from in-house breeding stocks at the University of Edinburgh housed under standard SPF conditions. All experiments were performed in accordance with UK Home Office and institutional guidelines.

2.2. Tissue processing and quantitative fluorescent western blotting

The spinal cord was dissected and immediately frozen on dry ice. Tissue and protein extracts were stored at −20 °C or −80 °C for 1, 7 days (d) or 4, 8, 12, 16, 20, 24 weeks (wk). Samples from 3 animals were used for each timepoint and storage parameter examined. Protein extracts were prepared from frozen tissue on the day of harvest. Protein extracts were prepared in RIPA buffer (ThermoScientific) with a protease inhibitor cocktail (Sigma). Tissue was defrosted in RIPA buffer on ice, homogenised using a motorised pestle, then spun at 14,000 rpm at 4 °C for 30 min. Extracts were removed and quantified alongside freshly collected samples. Quantitative fluorescent western blots were performed using 25 μg protein with primary antibodies against SMN (MANSMA12, mouse, 1:100; gift [26] and BD-SMN, mouse; 1:500; BD Transduction Labs) and COXIV (mouse; 1:2500; Abcam). Odyssey secondary antibodies were added according to the manufacturers' instructions (goat anti-mouse IRDye 680). Blots were imaged using an Odyssey Infrared Imaging System (Li-COR, Biosciences) at a resolution of 169 μM. Each blot was scanned and measured in triplicate to minimise user variability. Each sample was normalised against a COXIV loading control and then normalised to levels obtained in freshly prepared samples (harvested on the day of the experiment).

2.3. Statistical analysis

All data were collected using Microsoft Excel and analysed using GraphPad Prism software. For all statistical analyses, $p < 0.05$ was considered to be significant. All data are expressed as mean ± SEM.

3. Results

3.1. COXIV protein levels are stable over time

To establish that we could reliably quantify and compare protein levels across multiple samples after varying times in storage we carried out preliminary analyses of COXIV protein, a mitochondrial marker routinely used as a normalising control (actin and tubulin were not used as they are known to be altered in SMA tissue; [27]). Samples were stored at −20 or −80 °C. Although mitochondrial dysfunction has previously been reported in SMA, in our hands COXIV protein levels remained stable in spinal cord from SMA mice [27] and were both qualitatively (Fig. 1A) and quantitatively (Fig. 1B) preserved at all time points examined, regardless of storage temperature.

3.2. Detectable SMN protein levels decline over time in storage

We initially compared SMN levels in tissue stored for 1, 7 d or 4, 8, 12, 16, 20, 24 wk at −20 °C or −80 °C using both commercially-available and academically-available

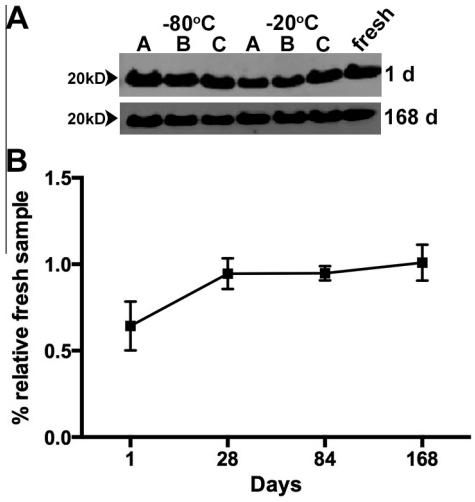

Fig. 1. COXIV protein levels remain stable over time in storage. (A) Representative western blots demonstrating stable COXIV protein levels independent of temperature after 1 d and 168 d (24 week) of storage (blots were prepared from samples from 3 individual mice "A-C" stored as frozen tissue and were compared to a freshly prepared sample "fresh"). (B) Graphical representation of stable COXIV levels when stored at −20 °C at 1, 28, 84 and 168 d, expressed relative to levels of COXIV in freshly harvested samples ($N = 3$ mice per timepoint; samples normalised against fresh = 1).