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The influence of storage parameters on measurement of survival of 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 of 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 a 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 SMN stability remains to be determined. We assessed murine SMN protein stability 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. We demonstrate that storage of samples at lower temperatures (-80°C), and as intact tissue, led to significantly better preservation of SMN immunoreactivity. However, considerable deterioration in SMN levels occurred even under optimal storage conditions. These findings need to be taken into consideration for both the design and interpretation of pre-clinical and clinical SMA studies where SMN protein levels are being used as a determinant of therapeutic efficacy.

1. Introduction

Proximal spinal muscular atrophy (SMA) is a leading genetic cause of infant mortality in humans, occurring with an incidence of 1 in 6000 live births [1]. SMA is an autosomal recessive disease caused by homozygous deletion or mutation of the ubiquitously expressed survival of motor neuron 1 (*SMN1*) gene [2]. In humans, there are two *SMN* genes, *SMN1* and *SMN2*. The *SMN2* gene produces considerably less SMN protein than the *SMN1* gene meaning that it cannot fully compensate when full-length SMN protein from the *SMN1* gene is lost [3, 4]. In its most severe forms, disease onset occurs before six months of age with death from respiratory distress usually within 1-2 years. 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.

There is currently no successful treatment or cure for SMA. However, several novel therapeutic approaches are showing promise in pre-clinical studies, such as gene therapy [5-9], anti-sense oligonucleotides [10-14], histone deacetylase (HDAC) inhibitors [15-19], and beta-catenin inhibitors [19]. Indeed, several of these approaches are entering clinical trials in patients.

In the absence of an established panel of robust SMA biomarkers [20], SMN levels are often used as a major biological read-out to evaluate therapeutic efficacy [5, 14, 21]. However, proteins can be unstable when outside their native environment. If this scenario were applicable to SMN, it is possible that patient or animal tissue samples obtained at different times and/or processed and stored in different ways (e.g. as is likely to occur in a multi-centre clinical trial on patients) could show differing levels of SMN protein as a consequence of different handling protocols rather than due to differences in SMN levels *per se*. As far as we are aware, no prospective studies evaluating levels of SMN stability and preservation under different tissue preparation and storage conditions have been reported. Here, we attempted to address this potentially important issue by analyzing frozen tissue samples and protein extracts stored at two standard storage temperatures (-20 °C and -80 °C) covering a period of 6 months. SMN protein levels were evaluated across a range of time points using both commercial and academically-available antibodies.

2. Materials and Methods

Animal models. Young adult wild-type CD1 mice were obtained from in-house breeding stocks at the University of Edinburgh. All mice were housed within the animal care facilities in Edinburgh under standard SPF conditions. All animal procedures were performed in accordance with UK Home Office and institutional guidelines.

Tissue processing and quantitative fluorescent western blotting. Fresh spinal cord tissue 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 independent 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 from frozen tissue in RIPA buffer (ThermoScientific) with a protease inhibitor cocktail (Sigma). Tissue was defrosted in RIPA buffer on ice, homogenised using a

motorised pestle, then immediately placed in a 4 °C centrifuge and spun at 14000 rpm for 30 minutes. Protein extracts were removed and stored on ice until analyses (< 1 hr). Prior to analyses, protein extracts were quantified alongside a freshly collected tissue sample. Quantitative western blots were performed using 25µg protein, primary antibodies against SMN (MANSMA12, mouse, 1:100; gift [22] 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).

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 \pm SEM.

3. Results

COXIV protein does not degrade over time.

To establish that we could reliably extract, store and quantify protein levels across multiple samples we carried out preliminary analyses of COXIV protein, a mitochondrial marker routinely used as a normalising control. Samples were stored as intact tissue or protein extracts for selected time points (1 d, 4 wk, 8 wk, 24 wk) at -20 and -80 °C, before quantifying COXIV levels. We demonstrate that COXIV levels were both qualitatively (Fig. 1A) and quantitatively (Fig. 1B) preserved from 1d up to at least 24 wk at -20 and -80 °C. From our initial data, we can conclude that western blotting methodology is suitable for such prospective analyses and that COXIV is an appropriate loading control to evaluate SMN protein levels over a similar 24 wk time period.

Detection of SMN protein with commercial and academic primary antibodies.

To establish whether different primary antibodies used to measure SMN protein levels varied in their ability to detect SMN protein, we initially compared SMN levels from tissue and protein extracts stored for selected time points (1, 7 d or 4, 8, 12, 16, 20, 24 wk) at a standard storage condition (-20 °C) (Fig. 2). There was no significant difference in the levels of SMN protein detected at any time point using either the commercially-available or academically-available SMN antibodies tested in this study (BD Transduction Laboratories and MANSMA12 [22]) (Fig. 2B). Both antibodies detected similar levels of SMN, regardless of how the tissue had been processed, with both antibodies revealing a robust decline in measured SMN protein levels of a period of 24 wk in storage (Fig. 2A and B).

Detectable SMN levels reduce after 7 days storage.

To determine whether SMN levels degraded similarly under different storage conditions, we first compared the effects of storage at -20 °C compared to -80 °C. After 24 wk, levels of SMN were visibly reduced at both storage temperatures, with a greater degradation observed when stored at -20 °C (Fig. 3A). The level of SMN protein detectable by quantitative western blotting was reduced after only

7 d of storage at both -20 °C and -80 °C (Fig. 3B). A particularly high decrease in SMN levels was observed in samples stored at -20 °C, particularly between 1 d and 7 d (from 77.5 % of levels detected in fresh samples at 1 d down to 35.8 % at 7 d). A smaller decrease was detected during the same time period with samples stored at -80 °C (88.7 % down to 76.3 % at 1 d and 7 d respectively). Detectable levels of SMN protein continued to reduce over time under both storage conditions, but the greatest overall drop was observed in samples stored at -20 °C (Fig. 3B). Indeed, there was a significant difference in levels of detectable SMN from samples stored at -20 °C and -80 °C at the majority of time points from 7 d onwards, with SMN levels more stably preserved at -80 °C (Fig. 3). At all time points examined, samples stored at -80 °C retained more than 50 % of SMN levels observed in fresh samples.

Extended preservation of SMN when samples are stored as frozen tissue.

Samples stored at -20 °C originating from both frozen tissue and protein extracts retained less than 50% of the SMN levels detectable in a fresh sample by 7 d (protein extract) and 4 wk (frozen tissue) (Fig. 4A). Although variable between time points, storage of samples as frozen tissue at -20 °C retained higher levels of SMN than samples stored as protein extracts. This was significantly different at the majority of time points tested (Fig. 4A). When stored at -80 °C, SMN levels were preserved above 50 % until at least 12 wk (protein extract) and 24 wk (frozen protein) (Fig. 4B). SMN levels from frozen tissue were higher than in protein extracts stored for the same time (Fig. 4B). At the longest time point tested (24 wk) SMN levels were highest in frozen tissue stored at -80 °C, (88.5 % of fresh) compared to 27.7 % in protein extracts.

4. Discussion

Here, we have demonstrated that detectable levels of SMN protein in *in vivo* samples decline over a fairly short period of time, regardless of the tissue handling protocols employed or the temperature used for storage. However, we also established that SMN protein remained more stable in samples stored as whole tissue rather than as extracted protein, with clear benefits of storing at -80 °C over -20 °C.

There is no cure for SMA, however, a number of novel therapeutics that are currently undergoing pre-clinical testing or clinical trials aim to affect disease progression by increasing levels of SMN protein. SMN levels are routinely measured to determine the efficacy of such therapeutics; however, given the instability of SMN demonstrated here, it is crucial to have an informed understanding of the degradation process to instigate best practise in terms of tissue collection and storage prior to analyses, thus improving study design and accurate interpretation of SMN level data. We propose that to maintain the highest possible levels of SMN protein from biological tissue samples it is best to flash freeze samples at a temperature of at least -80 °C and perform experiments to determine protein levels as quickly as possible after tissue sampling.

To minimise inter-sample variability and improve comparative analyses in preclinical or clinical trials, we suggest that if all required samples cannot be collected at the same time (as is often in the case in large pre-clinical animal studies and multi-centre clinical trials), then an equal number of control and SMA samples should be collected in parallel and stored identically. Our findings

suggest that comparative analyses should preferentially be carried out on samples collected at the same time to minimise differential SMN levels occurring due to differential SMN degradation associated with tissue processing and/or storage.

Overall, our findings highlight precautions that should be taken when using SMN protein levels as a therapeutic readout and/or biomarker, and highlight the importance of understanding sample handling and storage parameters for designing appropriate pre-clinical and clinical studies for SMA.

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Author Contributions

GH, HRF and THG conceived and designed the study; GH, SLR and ES carried out the experimental work; THG and GH analysed data; all authors contributed to the writing of the manuscript.

Figure legends

Fig. 1. COXIV levels are stable over 24 wk. (A) Representative blots demonstrating stable COXIV protein levels independent of storage temperature at 1 d, 28 d (4 wk), 84 d (12 wk) and 168 d (24 wk) (all blots prepared from samples stored as frozen tissue and using BD antibody). (B) Graphic representation of stable COXIV levels at 1, 28, 84 and 168 d, expressed per blot relative to levels of COXIV in a freshly harvested protein sample.

Fig. 2. Identical performance of academic and commercially available SMN antibodies. (A) Representative blots of SMN levels detected at 1 d, 56 d (8 wk) and 168 d (24 wk) using two SMN antibodies (BD, BD Transduction Laboratories and MANSMA12) (all blots prepared from samples stored as frozen tissue). (B) Graph demonstrating that identical levels of SMN are detected with MANSMA12 and BD SMN antibodies from 1 d to 168 d at -20 °C (all tests twoway ANOVA; N > 3 mice).

Fig. 3. Storage at lower temperatures improves preservation of SMN levels. (A) Representative blots demonstrating the effect of storage temperature (-20 °C vs -80 °C) on SMN levels at 1 d and 168 d (24 wk) (all blots prepared from intact tissue using MANSMA12 antibody). (B) Storage of samples at -80 °C preserved SMN levels above 50 % (grey horizontal line) for a longer time period than storage at -20 °C (** P < 0.001; **** P < 0.0001; all tests two-way ANOVA; N > 3 mice).

Fig. 4. Storage of sample as tissue over protein extracts increases longevity of SMN preservation. (A) After 7 d storage at -20 °C less than 50 % (grey horizontal line) SMN levels remained detectable. (B) Storage of tissue rather than protein extracts consistently improved preservation of SMN levels (> 50 %) from 1 d to 168 d (24 wk) at -80 °C (* P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001; all tests two-way ANOVA; N > 3 mice).

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