TREAT-NMD Activity A07: Accelerate preclinical phase of new therapeutic treatment developement

Work package 7.4: Develop standardised protocols and procedures for harmonising and accelerating pre-clinical studies (including standardised data analysis)

SOP Title	**Determination of hydroxyproline content as a measure of fibrosis in nondystrophic and dystrophic skeletal muscle.**
SOP (ID) Number	M.1.2_006
Version	1.0
Status	Final/ November 25th 2009
Last reviewed	November 25th 2009
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1 OBJECTIVE

The objective is to determine the total hydroxyproline expressed per mg of muscle tissue wet weight (hydroxyproline content) as a quantitative measure of collagen deposition and fibrosis. The procedure is adapted from Prockop and Udenfriend (1960) and Switzer and Summer (1971). Briefly, individual muscles are weighed before being acid-hydrolyzed at 130°C for 12 hours in 5N HCl (10 mg muscle wet weight/ml). Samples of the hydrolysate equivalent to 0.5 mg of muscle (50 µl) are diluted with 2.25 ml of distilled water and neutralized with appropriate amounts of 0.1N KOH (phenolphthalein indicator). Sodium borate buffer (0.5 ml, pH 8.7) is then added and the mixture is oxidized with 2.0 ml of 0.2 M chloramine-T solution. After a brief incubation (25 minutes), the oxidation reaction is stopped by adding 1.2 ml of 3.6 M sodium thiosulfate. Since the pyrroline and pyrrole carboxylic oxidation products that are formed from hydroxyproline are not soluble in toluene, contaminating impurities are extracted by adding 2.5 ml toluene and saturating amounts of KCl (1.5 g). After appropriate mixing of the toluene and aqueous phases, the phases are separated by centrifugation (300 to 400g for 1 minute). The toluene phase containing the extracted impurities is then removed and discarded. The remaining aqueous layer containing the hydroxyproline products is heated for 30 minutes in boiling water to convert the oxidation product of hydroxyproline, pyrrole-2-carboxylic acid, to pyrrole. The final pyrrole reaction product is then removed in a second toluene extraction, and 1.5 ml of the final toluene layer is mixed with 0.6 ml Ehrlich's reagent for colorimetric assay against hydroxyproline standards (0.0, 0.5, 1.0, 2.0, 4.0 6.0 µg hydroxyproline) at 560 nm. Hydroxyproline contents of individual muscles are expressed as µg hydroxyproline/mg muscle wet weight.

2 SCOPE AND APPLICABILITY

The method provides a quantitative scalar determination of the total amount of hydroxyproline per mg muscle wet weight. The technique was first used to assess the hydroxyproline levels in the mdx diaphragm (Stedman et al., 1991) and provides a simple and relatively rapid quantitative determination that can be used to assess efficacy in reducing fibrosis in dystrophic muscle.

Under some circumstances, this protocol for determining hydroxyproline content should be complemented by independent histological determinations of fibrosis using a trichrome staining technique (or suitable alternative) and point counting measures to assess the proportion and distribution of fibrotic tissue within muscle cross sections. For example, in some cases a particular drug or treatment may not alter the total hydroxyproline expressed per mg of muscle, but may nevertheless alter the distribution of collagen within the muscle. In such cases, point-counting procedures for assessing the proportion of collagen in muscle cross-sections and morphometric evaluation of the density and area of collagenous fibrotic plaques provide an independent measure to exclude the possibility that a treatment alters collagen distribution without altering total collagen expression.

3 CAUTIONS

Age, Muscle, and Gender Considerations. Since collagen levels increase with age in skeletal muscle from both nondystrophic and dystrophic mice, it is imperative that comparisons be made only between groups that are appropriately age-matched. Collagen levels also differ between different muscles in both nondystrophic and mdx mice. It is therefore quite important to identify the muscle being evaluated. Experiments comparing hydroxyproline contents in adult gastrocnemius and costal diaphragm muscle indicate that hydroxyproline content is not significantly elevated over nondystrophic levels in the gastrocnemius, but is quite substantially elevated in the costal diaphragm (Graham et al., 200X; Fig. 2). These results and those of Stedman et al (1991) clearly identify the costal diaphragm as the most appropriate muscle to use when examining the potential efficacy of experimental treatments to reduce fibrosis in the mdx mouse. We have not observed obvious gender differences in hydroxyproline content in either nondystrophic or mdx mice.

Purity of muscle sample. In determining hydroxyproline content, it is essential that only muscle tissue be used, and that all tendinous material be carefully removed from the sample before performing the assay. A good sample would be obtained by using approximately ½ of an entire costal hemidiaphragm. The muscle should be flash-frozen and may be stored at -80^{O} C for several weeks before running the assay. In practice, samples are usually run within about 4 weeks of the time that they are isolated.

Critical steps in the hydroxyproline assay. There are some particularly important steps in the hydroxyproline assay that must be monitored carefully to ensure quality results.

(Steps 2A and B).The first two steps in the hydrolysis (step 2A, B in Methods) of the muscle tissue involve overnight incubation of the sample in 5 N HCl at 130°C. The acid solution must be contained within stoppered glass tubes that do not leak. Therefore, the volume of acid in each tube should be visually monitored before and after the incubation period to ensure it remains constant throughout the incubation period. Instances in which there is a clear loss of volume indicate a gas leak and should result in termination of the experiment for that sample. To avoid this problem, use brand new screw-tops and make sure that the screw tops are tightly applied to each tube prior to the incubation.

(Step 4N). In removing the toluene layer containing the final pyrrole reaction product, it is imperative not to disturb or remove any of the aqueous layer. If aqueous solution is removed, it clouds the colorimetric reaction measured in step 5. To avoid this problem, remove only 1.5 ml of the 2.5 ml toluene layer and make the appropriate correction to determine the hydroxyproline level in the 2.5 ml of toluene extract (Evaluation and Interpretation of Results).

Continuity of assay. Theoretically, once the muscle sample is fully hydrolyzed (Step 2c), it may remain at room temperature for up to 3 to 4 hours before processing the sample (Step 4). Once the processing steps have begun (Step 4A), they should continue without interruption until the assay has been completed (Step 5). In practice, it takes about 5 hours to fully process about 30 test tubes and the process is facilitated by having more than one investigator engaged in Steps 4 and 5.

Precautions. Standard protection (gloves, eye protection, lab coat) should be used at all times when handling acids and toluene solutions. Toluene extractions should be performed under a fume hood. All solutions at the end of the procedure should be discarded using environmentally-appropriate procedures for handling acids and organic solvents.

4 MATERIALS

1) Stock Buffer Composition:

50 g citric acid monohydrate (Sigma #C7129)
12 ml glacial acetic acid (Sigma #A6283)
120 g sodium acetate trihydrate (Sigma #S7670)
34 g NaOH
Total volume in ddH_2O (dd means double distilled or filtered and distilled) = 1 liter

Measure 900 ml ddH_2O and add 12 ml glacial acetic acid slowly while mixing. Add citric acid monohydrate, sodium acetate trihydrate, and NaOH. Add ddH_2O to total volume of 990 ml and adjust pH to 6.0 with HCl and NaOH as needed. Add ddH_2O to total volume of 1 liter and check pH again. Label (HP Stock Buffer solution, pH, initials, date) and store in refrigerator.

2) Ehrlich's Reagent (from Prockop and Udenfriend, 1960; for step 4P):

Slowly mix 27.4 ml of concentrated sulfuric acid to 200 ml of absolute alcohol in a 500 ml beaker.
In another beaker, add 120 g of p-dimethylaminobenzaldehyde to 200 ml of absolute alcohol.
Slowly mix the two solutions in the first beaker.
Store the final solution in the refrigerator, and allow crystals to come out of the solution before using.

Other chemicals needed:

Phenolphthalein solution (1%)
0.8 N and 0.1N KOH
0.1 M sodium borate buffer (pH 8.7)
0.2 M chloramine-T
3.6 M sodium thiosulfate
KCl
Toluene

Instruments needed for measurements:

Glass test tubes (15 ml capacity) with screw caps
Oven
Spectrometer
Tabletop centrifuge
Tabletop test tube Invertor
Tabletop vortexor

5 METHODS

1) Tissue preparation

Blot and weigh fresh or thawed muscle sample (e.g. ½ to 1 costal hemidiaphragm) to obtain wet weight. Make sure that the muscle sample is carefully trimmed and contains no tendinous material.

2) Hydrolysis. Always wear appropriate protection (lab coat, heavy rubber gloves, eye protection) and use standard safety procedures when handling acids.

A. Add 5N HCl (1ml HCl /10mg tissue) to the muscle sample in glass test tube. Tighten screw-tops carefully and ensure that the screw-tops are not over-worn from previous use.

B. Incubate tubes at 130^{o}C for 12 hrs (setting is number 13 on the Fisher Scientific Isotemp oven).

C. Remove muscle samples from oven and cool to room temperature. Do not open the test tubes until they are cooled to room temperature.

3) Prepare chemicals and solutions

A. Determine how many tubes (n) will be run. This includes all the experimental samples (in triplicate) and the hydroxyproline standards (in duplicate) plus the blank tube.

B. Weigh approximately 1.5 g of KCl for each test tube and place in weigh boats (see step 4G).

C. Prepare chloramine T solution (for step 4E):

1) Determine the total volume of chloramine T solution that will be needed. This is equal to 2n +2 in ml (extra 2 ml for making sure there is enough volume).

2) Weigh out enough chloramine T for the total volume that is required. Based on the molecular weight of chloramine T (Sigma # C9887; 227.6 g/mole):

(2n +2) ml x 0.2 moles/1000 ml x 227.6 g/mole = Amount Chloramine T needed in g.

Example: for 10 tubes to be run:
(20 +2) ml x 0.2 moles/1000ml x 227.6 g/mole = 1.00 g chloramine T

3) Dissolve chloramine T in the appropriate volume of buffer:

a.) Prepare the following volumes:

Volume of Stock Buffer (see Materials) = 0.5*(2n +2)
Volume of ddH_2O = 0.2*(2n+2)
Volume of methylcellosolve (2 methoxyethanol; Sigma #284467) = 0.3*(2n+2)

b.) Instructions for preparing solution:

Add the appropriate volume of ddH_2O to the amount of chloramine T determined in step C2, and then the appropriate volumes of the stock buffer and methyl cellosolve. Mix thoroughly.

4) Sample and standard processing. To ensure safety, all of the following steps should be conducted using a fume hood and standard laboratory safety precautions required for handling acids and organic solvents.

A. Make sure hydrolyzed muscle sample at room temperature is mixed well (invert closed tube to mix). Remove 50 µl of the hydrolyzed muscle sample, which represents 0.5 mg of the original muscle wet weight, and add to 2.25 ml of ddH_2O (need total volume of 2.3 ml) in glass test tube. Repeat in triplicate for each muscle sample. In a test tube marked "0" (blank), add 2.3 ml ddH_2O. Screw a cap on each test tube.

B. Set up the hydroxyproline standards in 2.3 ml of ddH_2O (run each in duplicate) containing, for example, 0.75, 1.5, 3.0, and 6.0 µg of hydroxyproline. A 1mg/ml stock solution of hydroxyproline (in ddH_2O) may be stored in the freezer. Screw a cap on each test tube.

C. Add 1 drop of phenolphthalein solution (1%) to each tube and adjust pH to a "faint pink color" with 0.1 N KOH (Using a Pasteur pipette, add about 5 drops of 0.8 N KOH then add drops of 0.1N KOH until faint pink).

D. Add 0.5 ml of 0.1 M sodium borate buffer (pH 8.7) to each tube, screw a cap on each tube, and mix by vortexing at low speed.

E. Add 2.0 ml of freshly prepared 0.2 M chloramine-T solution to each tube, screw a cap on each tube, and incubate at room temperature for 25 minutes.

F. Add 1.2 ml of 3.6 M sodium thiosulfate to each tube, screw a cap on each tube, and mix thoroughly (vortex) for 10 sec.

G. Add 1.5 g KCl and 2.5 ml toluene to each tube. Screw a cap on each tube.

H. Shake sample for 5 minutes or slowly invert test tubes 100 times, preferably using a test tube invertor.

I. Centrifuge at approximately 300-400 g (1 minute) to separate layers.

J. Remove toluene phase using clean Pasteur micropipette. Discard using appropriate procedures for organic solvents.

K. Heat remaining aqueous phase in boiling water for 30 minutes (cap tightly).

L. Cool to room temperature.

M. Add 2.5 ml toluene to each tube, screw a cap on each tube, and repeat steps 4H-I.

N. Remove 1.5 ml of toluene layer and place in a separate test tube. Screw a cap on each tube.

O. Gently warm stock Ehrlich's reagent (see Materials) that is kept in refrigerator in order to remove crystals.

P. Add 0.6 ml Ehrlich's reagent, mixing well to prevent layering of the solvent. Incubate at room temperature for 30 minutes.

5) Measure samples

Using a conventional glass or quartz cuvette, measure the blank-corrected (sample – blank) absorbance (560 nm) for each solution obtained in step 4P.

6 EVALUATION AND INTERPRETATION OF RESULTS

A. Perform a linear regression analysis of the results using the hydroxyproline standards to identify the relationship between hydroxyproline (µg) and Absorbance (e.g., Fig. 1).

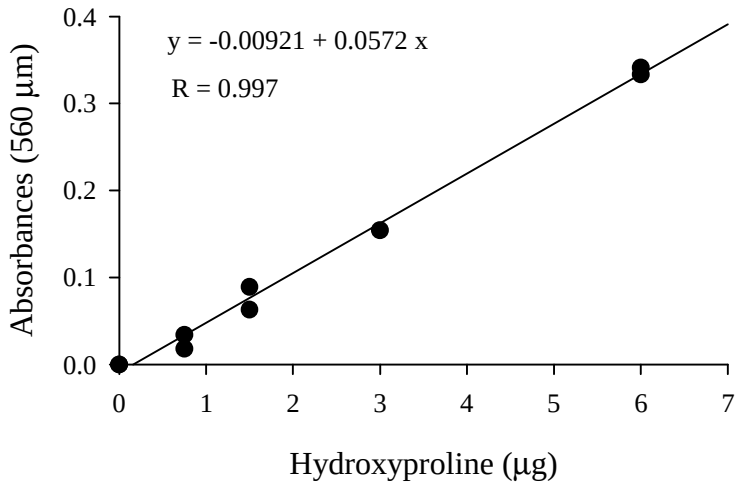

Figure 1. Example of a hydroxyproline standard curve and the least squares determination of the standard curve equation relating hydroxyproline (µg) to Absorbance. Inset shows the results of regression analyses.

B. Determine the amount of hydroxyproline (µg) in each sample test tube (Step 4P) using the regression curve from the hydroxyproline standards. In the example shown (Fig. 1), the appropriate relationship is:

Sample Hydroxyproline (µg) = (Sample Absorbance + 0.00921)/0.0572

C. Since the amount of hydroxyproline in the final colorimetric reaction (Step 4P) represents a proportion (1.5 ml/2.5 ml) of the total hydroxyproline in the final toluene extract (Step 4M), multiply the result obtained in Step 6B by (2.5/1.5) to obtain the total amount of hydroxyproline present in the final extract.

D. Divide the result obtained in Step 6C by the amount of muscle (wet weight) contained in the initial sample (0.5 mg; Step 4A) to obtain the hydroxyproline content (µg hydroxyproline/mg muscle). Examples of typical values obtained from the costal diaphragms of 14 month old nondystrophic and mdx mice are shown (Fig. 2).

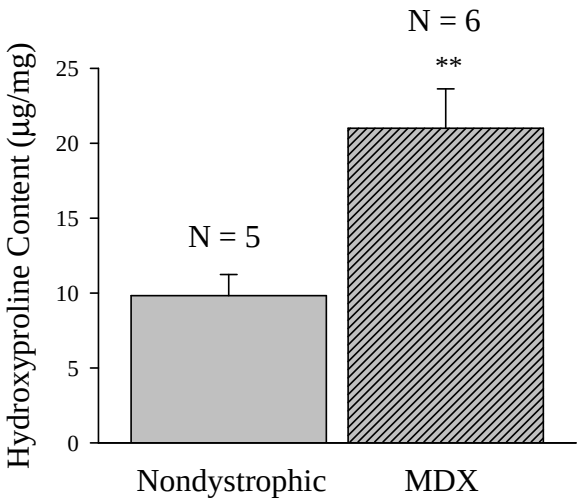

*Figure 2. Hydroxyproline Contents (µg hydroxyproline/mg muscle) determined from the costal diaphragms of 14 month nondystrophic (gray histobar) and mdx (gray hatched histobar) mice. N is the number of preparations (mice). Shown are the means and standard errors. ** indicates p< 0.01 between mean nondystrophic and mdx values.*

7 REFERENCES

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