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Urine hydroxyproline correlates with progression of spasticity in cerebral palsy

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ABSTRACT

Background: Most Cerebral Palsy (CP) patients develop muscle spasticity which is characterized by jerky movements and muscle and joint stiffness. This increase of muscle stiffness in spastic CP has been correlated with the accumulation of collagen in the muscle as detected by the increase in muscle hydroxyproline, a major component of collagen.

Objective: The objective of the study is to determine if there is any correlation between muscle and urine hydroxyproline levels in spastic CP. Further, to determine if Urine Hydroxyproline levels are different between spastic CP with and without contracture. Finally to determine if UH levels can be correlated with severity of CP as determined by Modified Ashworth Scale (MAS) and Gross Motor Function Classification System (GMFCS) scores.

Methods: This was a cross sectional comparative study, conducted in the tertiary hospital, Malaysia from June'2012 to December'2014. Children with spastic CP (6 to 18 years) who were scheduled for muscle/tendon lengthening as part of the on going management and children with pure spasticity were included in this study. Normal children who are aged and sex matched to the CP children were included. Muscle biopsy and urine samples were collected for MH and UH analysis respectively.

Results: A total of 48 children, aged 6 to 18 years (17 normal; 16 spastic CP without contracture, 15 spastic CP with contracture) were included in this study. Muscle biopsy (only for CP children with contracture) and urine samples were collected. A significant negative correlation was noted between the MH (261.894±69.077ng/ml) and UH (13.266±7.999ng/ml) levels (p=0.031). There was a statistically significant correlation between UH levels and the MAS score (p=0.01), and GMFCS score (p=0.015).

Conclusion: UH quantification may be an objective tool to estimate the severity and progression of spasticity in CP.

Keywords: cerebral palsy, hydroxyproline, muscle spasticity, collagen, contracture

INTRODUCTION

Cerebral palsy (CP) is described as a movement disorder caused by upper motor neuron lesions in the developing brain. It occurs at a rate of 3.6 per 1,000 live births (1). The spastic type has been identified as the most common type of cerebral palsy. The hallmark of muscle spasticity is persistent stimulation with a reduction in the inhibition of the stretch reflex. If this mechanism continues unchecked, then excess collagen accumulates, leading to fibrosis and contracture.

A study conducted by Booth et al (2) revealed increased collagen levels in spastic muscle. In addition, the amount of total collagen, which increases with the severity of the disorder, can lead to contracture. Contracture is an adaptation of the muscle that limits the range of movement around the joint. The muscle contracture that develops among CP patients is a poorly understood adaptive mechanism (3). Abundant extracellular matrix (ECM) and fibrosis has been noted within the contracted muscles of patients with cerebral palsy (2). Collagen that accumulates in the spastic muscle endomysium is thick and fibrotic, especially in severe cases, which suggests that collagen may increase the muscle stiffness in spasticity (2). De Bruin et al. questioned the belief that the endomysium is affected in CP muscle (PLOS one 2014) instead highlighting the role of thickening of the tertiary perimysium (4). A study conducted in children with spastic diplegia noted a predominance of type 1 fiber in their spastic muscles (5). Another study on the histological findings of contracted hamstring muscles in children with spastic CP reported that ECM stiffening is correlated with increased collagen and in

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vivo sarcomere length, which leads to high passive stress (6, 7). Moreover, spasticity-induced fibrosis has been shown to cause limitations in longitudinal growth (8).

Hydroxyproline is a major component of the collagen protein. Hydroxyproline quantification has been used as an indirect measure of collagen in the spastic muscles of children with CP, and its muscle hydroxyproline (MH) level correlates with the severity of spasticity (2). The distinctive feature of collagen is the regular arrangement of amino acids, which creates a long, fibrous structured protein. The amino acid sequence consists of Gly-Pro-X or Gly-X-Hyp, where X may be any amino acid residue, with the Gly–Pro–Hyp motif occurring frequently. Collagen contains a high concentration of 4-hydroxyproline (Hyp). In addition, up to 14% of dry weight collagen is composed of Hyp. In contrast, only 1% Hyp is found in elastin. The human body is capable of self-synthesizing glycine and proline but not hydroxyproline. Hydroxyproline is a modified amino acid exclusive to collagen. Therefore, its proportion to other amino acids is constant, and estimate of its concentration is directly related to the amount of collagen within a tissue (9). Increased collagen as well as other ECM components were observed in spastic muscles by immunohistochemistry. The organization of collagen may be altered in CP to create a stiffer ECM. Total collagen is measured by assaying for the exclusive collagen-specific modified amino acid hydroxyproline (10). Hydroxyproline is metabolized, excreted, and removed from the lung and kidney. Approximately 70% of the peptide released by collagen degradation is hydrolysed into its constituent amino acids, catabolized to carbon dioxide, and excreted via the lung. The other 30% is excreted via the urine. It has been suggested that the ratio of hydroxyproline excreted from the lung to that excreted from the urine is fairly constant; therefore, changes in collagen breakdown can be quantified by measuring the changes in the urine concentration of the prolyl-hydroxyproline peptide, which may reflect ineffective peptidase activity (10, 11, 12). However, no studies have investigated the correlations between the severity of clinical spasticity, Muscle Hydroxyproline (MH), and Urine Hydroxyproline (UH).

The MH level can be measured only via a muscle biopsy, which is an invasive procedure and is not realistic for monitoring spasticity. Some clinical tests are used to assess spasticity in cerebral palsy, including the Modified Ashworth Scale (MAS) (13) and the Modified Tardieu Scale and Gross Motor Function Classification System Scoring (14). These tests are subjective and often exhibit inter-observer variations. Thus, a complete assessment is essential to make a determination about treatment for spasticity in a child with CP (15).

It is known that the UH level reflects muscle collagen metabolism (16, 17). It has been reported that UH excretion is a sensitive indicator of collagen breakdown and can be used at the clinical level to predict changes in collagen metabolism (10). The aim of this study is to determine the correlation between the severity of clinical spasticity and UH and MH among children with CP who exhibit contracture or spasticity without contracture. UH may provide a non-invasive tool for monitoring the progression of spasticity and the efficacy of spasticity management.

METHODOLOGY

This cross-sectional comparative study was conducted in a tertiary hospital in Malaysia from June 2012 to December 2014. Children with spastic CP (quadriplegic, diplegic, and hemiplegic) were recruited from the pediatric orthopedic and rehabilitation clinic according to the inclusion and exclusion criteria. Children with spastic CP aged between 6 and 18 years who were scheduled for a surgical lengthening procedure (e.g., hamstring, Achilles tendon, hip adductor) as part of their ongoing treatment as well as children with spasticity without contracture were included. Patients with a previous history of neurectomy, recent soft tissue injury, or collagen disorders as well as patients who received a botulinum toxin injection in the previous 6 months were excluded from the study. Healthy children who were age and sex-matched to the study subjects were included as a control group. Informed consent was obtained from the parents to take a muscle biopsy from the site of the surgical lengthening procedure. Standard proforma was used to collect the demographic data and clinical assessments. The MAS (15) was used to assess spasticity, and the Modified Tardieu Scale (12) was used to differentiate spasticity from contracture. Gross Motor Function Classification System (GMFCS) was assessed to ascertain the severity of CP based on the basis of self-initiated movement abilities of the patients.

Urine Sample Collection and Storage

Urine samples were collected from the CP children with contracture or spasticity without contracture and the healthy control group. Patients were asked to abstain from all dairy products and gelatin for 24 hours before the urine sample collection for the measurement of urine hydroxyproline (UH) as these diets are rich in hydroxyproline content. The collected urine was transported at 4°C to the laboratory. Particulates from the urine were removed by centrifugation and

Table 1: Demographic data among children with CP

		CP children with Spasticity (n=16)	CP children with Contracture (n=15)
Spastic Diplegia		11	11
Spastic Quadriplegia		1	4
Spastic Hemiplegia		4	0
GMFCS Score	(I)	15	-
	(IV)	1	11
	(V)	-	4
MAS Score	(I)	16	-
	(II)	-	11
	(III)	-	4

the supernatant was stored at -20°C. The samples were centrifuged again to remove any additional precipitates that may have appeared during storage before the hydroxyproline assay.

Muscle Biopsies

A muscle biopsy of 0.5cm x 2.0 cm (approximately 100 mg) was taken from the hamstring muscle belly (away from muscular tendon junction) within 30 minutes of excision. A small section (approximately 0.5cm x 0.2cm) was formalin fixed for histological processing. Another section was snap-frozen in OCT embedding medium for cryosectiong followed by immunostaining with collagen type I (Abcam, USA) Secondary antibody used was goat anti-rabbit IgG–Alexa Fluor 594 (Invitrogen, USA). The remaining muscle biopsy was directly snap-frozen. The snap-frozen muscle was homogenized in 1 ml of phosphate buffer using a glass pipette in an Eppendorf tube. The homogenized tissue suspension was subjected to two freeze-thaw cycles to break the cell membranes. The homogenates were centrifuged for 5 minutes at 5000 rpm at 2-8°C to remove the tissue debris. The supernatant was removed for immediate assay of hydroxyproline.

Determination of Hydroxyproline Levels

ELISA was performed in triplicate according to the vendor's protocol (Cusabio Human Hydroxyproline Elisa Kit, CSB-E08837h). Briefly, hydroxyproline standards, urine and muscle lysate were added to a pre-coated microplate with antibody specific for Hydroxyproline. A biotin-conjugated antibody specific for Hydroxyproline was added followed by avidin-conjugated horseradish peroxidase (HRP). Finally, a substrate solution (TMB) was added to react with any bound HRP. The reaction was stopped with a Stop solution, and the intensity of the color was measured within 5 minutes using a microplate reader (Powerwave HT, BioTek Instruments Inc, USA) set to 450 nm. Readings at 540 nm were used for background subtraction.

Statistical Analysis

The data were analyzed using SPSS version 19. Data is presented as mean ± standard error of mean. The data were analyzed for normality, and non-parametric tests were chosen to compare the medians between groups to assess statistical significance.

RESULTS

Demographic data of patients is represented in **Table 1**. The mean GMFCS and MAS scores were higher for contracted CP patients (2.6 and 2.27, respectively) than that for spastic CP patients (1.75 and 1.06, respectively) (**Figure 1**). **Table 2** shows the comparison of MAS and GMFCS scores between CP children with contracture and spasticity. Mann-Whitney test revealed a significant higher median for GMFCS and MAS scores between the Contracted and Spastic groups ($p<0.05$). Muscle biopsies were derived from the normal region of the hamstring of children (n=3) undergoing surgery after traumatic hamstring injury.

Histological evaluation revealed an increase in muscle fiber diameter in the contracted muscles compared to that in the normal muscles (**Figure 2A-D**). Immunofluorescence staining of collagen type I did not reveal apparent difference in collagen distribution between contracted and normal muscle. (**Figure 2E, F**)

Mean UH level was higher in the contracture (56.38 ng/ml ± 43.13ng/ml) (n=15) compared with that in the spasticity (31.93 ng/ml ± 26.91ng/ml) (n=16) and normal (28.37 ng/ml ± 20.21ng/ml) (n=17) groups (**Figure 3**). Kruskal-Wallis test revealed that the differences in median between groups were not statistically significant ($p>0.05$) as shown in **Table 3**.

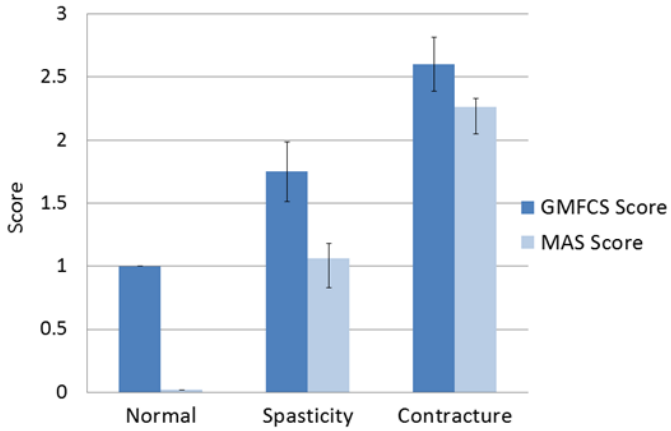


Figure 1: *Mean scores of GMFCS and MAS*

Table 2: Difference in MAS and GMFCS scores between CP children with contracture and spasticity

	Group	N	Median	Range	Mean Rank	Sig.
GMFCS	Contracted	15	2	1-4	8	0.014*
	Spasticity	16	2	1-4	8.5	
MAS	Contracted	15	2	2-3	8	0.000*
	Spasticity	16	1	1	8.5	

Mann-Whitney 2-tailed *Significant, p<0.05

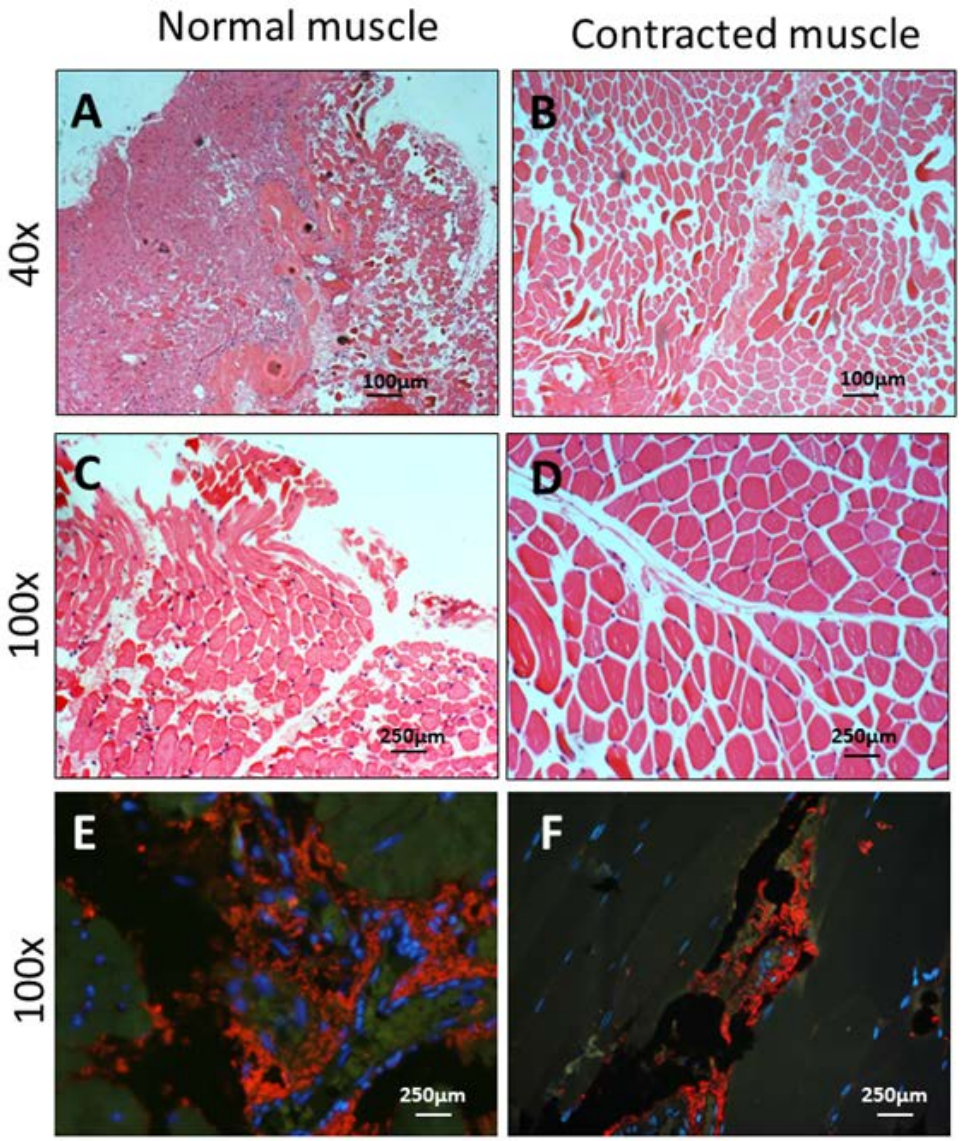


Figure 2: *Immunofluorescence staining of collagen type I*

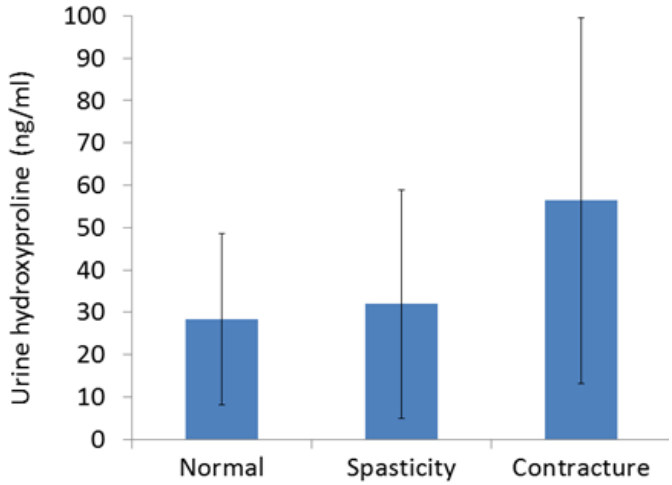
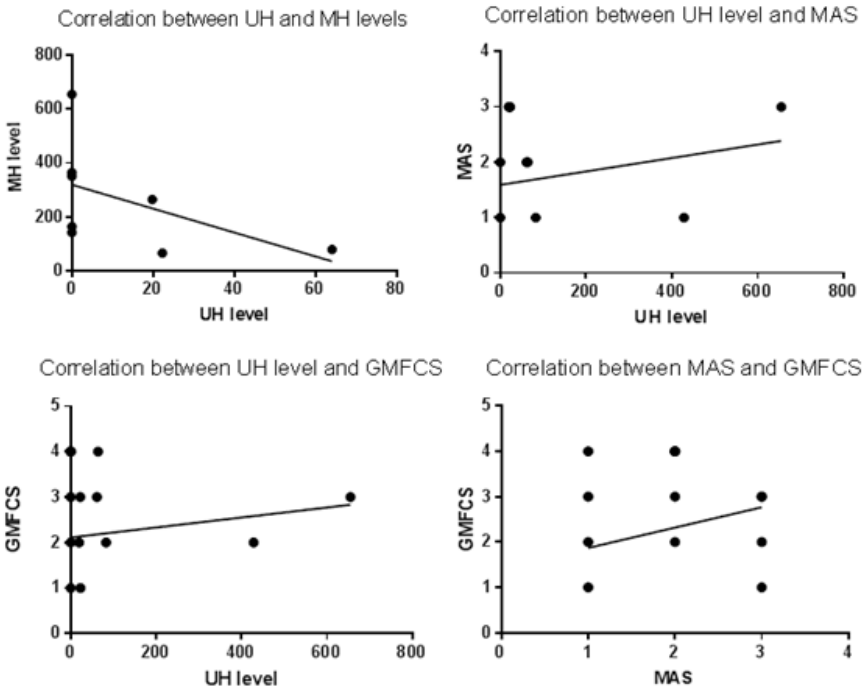


Figure 3: *Mean urine hydroxyproline level*

Table 3: Median of urine hydroxyproline levels among the study groups

	Group	N	Median	Range	Mean Rank	Sig.
Urine Hydroxyproline Level	Contracted	15	0	0-655	8.000	0.335*
	Spasticity	16	0	0-428	8.000	
	Normal	17	0	0-342	8.000	

Kruskal-Wallis *Not significant, p>0.05



	UH vs. MH	UH vs. MAS	UH vs. GMFCS	MAS vs. GMFCS
Spearman r				
r	-0.6819	0.388	0.2532	0.4106
95% confidence interval		0.02814 to 0.6589	-0.1219 to 0.565	0.05492 to 0.6738
P value				
P (one-tailed)	0.0387	0.0155	0.0847	0.0109
P value summary	*	*	ns	*
Exact or approximate P value?	Exact	Approximate	Approximate	Approximate
Significant? (alpha = 0.05)	Yes	Yes	No	Yes
Number of XY Pairs	8	31	31	31

Figure 4: *Correlation levels*

Table 4: *Comparison of median of UH and MH levels in the Contracted Patients*

Null Hypothesis	Test	Sig	Decision
The median of differences between UH and MH equals 0	Related samples Wilcoxon Signed Rank Test	0.12*	Reject the null hypothesis

Asymptotic significances are displayed. * Significant, p<0.05

Table 5: *Correlation between UH and MH among CP children with contracture*

Mean (ng/ml)		N		UH	MH
UH	13.266	8	Correlation coefficient	1.000	-0.682
			Sig. (1-tailed)	-	0.031*
			N	8	8
MH	261.894	8	Correlation coefficient	-682	1.000
			Sig. (1-tailed)	0.031*	-
			N	8	8

Spearman's Correlation Test *Correlation is significant (1-tailed), p<0.05

Table 6: *Correlations between MAS, GMFCS, and the UH level*

Mean (ng/ml)		N		UH	GMFCS	MAS
UH	38.3113	48	Correlation coefficient	1.000	0.253	0.388
			Sig. (1-tailed)		0.085	0.015*
			N	48	31	31
GMFCS	2.16	31	Correlation coefficient	0.253	1.000	0.411
			Sig. (1-tailed)	0.085	-	0.011*
			N	31	31	31
MAS	1.65	31	Correlation coefficient	0.388	0.411	1.000
			Sig. (1-tailed)	0.015*	0.011*	-
			N	31	31	31

Spearman's Correlation Test *Correlation is significant (1-tailed), p<0.05

Further analysis was performed to compare mean of MH (261.894ng/ml ± 69.077ng/ml) and UH (13.266ng/ml ± 7.999ng/ml) for patients in the Contracted group (n=8). Wilcoxon Signed Rank Test showed that median was significantly higher for MH than UH (p<0.05) in the Contracted group (**Table 4**).

Spearman's correlation test was performed to establish the correlation between the following variables: UH, MH, GMFCS and MAS Scores. An inverse correlation was found between UH and MH in the Contracted group (p<0.05) (**Table 5**) while a positive correlation was established between the UH and MAS Score, and between GMFCS and MAS Scores in CP patients (**Table 6**).

DISCUSSION

As hydroxyproline is a stable amino acid unique to collagen, its level in urine reflects the amount of collagen turnover in the body. It is important to keep in mind that the increase in urine hydroxyproline levels can be due to a few scenario: 1. An increase in collagen synthesis in the body. Hydroxyproline is released during the breakdown of pro-collagen aminoterminal propeptides during collagen synthesis (18, 19). 2. An increased in collagen degradation activity in the body. Hydroxyproline is liberated from the breakdown of collagen either due to direct tissue injury or an increased activity of matrix metalloproteinase (MMP), the enzyme responsible for collagen breakdown. An increase in collagen degradation can also be triggered as a response to the increased in collagen synthesis in order to achieve collagen homeostasis in the body.

The significantly higher amount of MH compared to UH in our study is to be expected. It was reported that about 90% of the hydroxyproline released by the breakdown of collagen in the tissues will eventually completely oxidized and catabolized in the liver to urea and carbon dioxide while the remaining 10% is excreted in urine without any further metabolism (20, 21).

In our study, there was an increase in UH levels in the CP patients compared to normal children and UH level was highest among CP children with contracture. This increasing trend of UH hydroxyproline from normal children to CP with contracture suggest a correlation with increase muscle tone. This is supported by our finding that UH levels were high in the group with high MAS scores (i.e., MAS≥2) (**Table 3**). The correlation between UH and MAS was statistically significant (p=0.001). Thus, UH may be used as an adjunct clinical tool to MAS scoring in assessing muscle tone.

MAS score in turn was positively correlated with GMFCS score in our study. It was noted in another study that patients with higher MAS scores in the contracted group had a restricted range of movement with limited mobility in the affected joint, which leads to a contracted muscle with reduced functional range (22). Previous studies reported that high MAS scores were also associated with an increase in sarcomere length and extracellular matrix (ECM) contents (2, 5, 23). It is likely that the accumulation of ECM within spastic muscle causes changes in the muscle's mechanical properties that may contribute either directly or indirectly to the development of contractures and secondary bony abnormalities, which is a major causative factor leading to the mobility problems that have been observed in CP(5,22). It was also shown in another study that a reorientation of collagen fibers was responsible for the increase stiffness in ECM (24). The increased sarcomere length could be due to a reduced number of satellite cells (cells that are responsible for muscle growth and neural drive) in CP patients leading to a reduction in the growth potential overstretching of the muscle (14).

The apparent increase in muscle fiber diameter in the contracted muscle was contrary to previous reports (6, 25). Similarly, no increase in collagen was noted in the contracted muscles via immunohistochemical visualization. This warrants further study with large sample number.

The amount of collagen within a tissue at any particular time is not necessarily directly related to its synthesis rate, as the breakdown of collagen is tightly controlled by a number of mechanisms, including the regulation of the activities of matrix-degrading enzymes such as MMPs (26). When collagen accumulation outweighs the breakdown, fibrosis and contracture is resulted. It has been hypothesized that the persistent stimulation of the stretch reflex activity that accompanies spasticity leads to the accumulation of collagen and eventually causes fibrosis and contracture. Interestingly, an inverse correlation was established between UH and MH levels within patients with contracture. This indicates a shift in collagen homeostasis in the muscle where increased collagen synthesis in the muscle is not balanced by collagen degradation. One possible reason for this is that the high collagen metabolism in this group of patients triggers a defective MMP regulation whereby collagen continues to be accumulated in the muscle leading to contracture.

It is also noted that muscle collagen undergoes post-translational modification. This process can prevent collagen degradation by forming a stable hydroxyl-pyridinoline cross-link (27). Future studies should identify the cross-link accumulation in patients who experience spasticity, as this may explain the increased stiffness in spastic muscles. Future studies also need to better understand the correlation between the increase in ECM and the efficacy of the different metalloproteinase enzymes among different CP patient groups.

CONCLUSION

UH may be used as an objective tool to monitor the progression of spasticity in CP and improve the efficacy of spasticity management. The inverse correlation between UH and MH levels within patients with contracture is worth further investigation. This study is a platform for future large-scale clinical studies implicating the use of UH to monitor the progression of spasticity in children with cerebral palsy.

STUDY LIMITATIONS

The small sample size was the major limiting factor in this study. It was difficult to obtain informed consent from parents of patient for the muscle biopsies. Hence, no muscle biopsies from CP with spasticity and only two normal muscle biopsies were successfully obtained. Moreover, normal muscle may not be truly 'normal' as they are derived from traumatic injured muscle. Secondary reaction to trauma may bring about changes to muscle architecture and collagen distribution.

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