The effect of Duchenne Muscular Dystrophy on Purkinje cell number in the mdx mouse

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Background: Duchenne muscular dystrophy (DMD) is an X-linked recessive disease which causes skeletal muscle wasting in males, resulting in premature death during their early to mid 20s. Males with DMD carry defects in the gene encoding for dystrophin, a protein important in ensuring sarcolemmal stability. Dystrophin has also been implicated in disruption to Purkinje cells in the cerebellum. This disruption to cerebellar Purkinje cells has been proposed to be involved in reducing the IQ of affected boys. **Aim**: To compare Purkinje cell number and distribution in mutant mdx and normal mice. **Methods**: Cerebellar slices from both mutant (n=4) and normal (n=4) mice were prepared and stained. The number of Purkinje cells in each slice was estimated by three different cell counting techniques. Counting methods were as follows: firstly, the actual number of Purkinje cells per lobe; secondly, a randomised estimate where five random sections of the Purkinje cells layer were selected, counted and averaged; thirdly, an estimated maximum possible count, where three segments from the Purkinje cell layer with the highest density of cells were used to estimate Purkinje cell population. **Results**: No statistical significance in Purkinje cell numbers between the two groups was found. However, there was a trend towards a decrease in the median number of Purkinje cells in the mutant group, particularly in lobules 3, 4/5, 6 and 10. **Conclusion**: The study findings suggest a decrease in Purkinje cell number in mdx mice. The small sample size of this study precludes definitive statistical analysis of Purkinje cell numbers in either group. These findings demonstrate a need for larger mouse-model studies to accurately assess differences in cell numbers between the two groups. Given that the greatest difference in cell numbers was demonstrated in lobules 3 and 4/5, the authors suggest that DMD may affect the cerebellum during the maturation of these lobules. Importantly, a reduced Purkinje cell population may be implicated in the intellectual morbidity in boys with DMD.

Introduction

Duchenne muscular dystrophy (DMD) is an X-linked recessive disease with an incidence of 1 in 3,500 male live births, making it the most common form of muscular dystrophy worldwide. [1,2] Dystrophin, a protein which has a role in sarcolemmal stability in skeletal muscles, is absent in patients with DMD. The resulting progressive muscular weakness eventually leads to death. Deficiency in dystrophin has also been shown in neurons in the cerebral cortex, cerebellum and hippocampal CA1–CA3 regions. [3]

The cerebellum is covered externally by grey matter which is divided into: (1) a molecular layer; (2) a Purkinje cell (PC) layer; and (3) a granular cell layer. [4] PCs integrate and relay their synaptic information to the deep cerebellar nuclei (DCN) and vestibular nuclei. The DCN then project to the cerebral cortex via the thalamus, mediating fine motor control and balance. [5] Our research focuses on the effects of the lack of dystrophin on the cerebellum, in particular, the PC population.

Methods

Animals and tissue preparation

This opportunistic study was performed with discarded cerebellar slices from the UNSW Stewart Head Laboratory. All experiments were conducted blind to the phenotype of the mice and the mice were either ten or eleven weeks old. Prior to receiving the tissues, the mdx and control mice were anaesthetised with halothane then decapitated with the individual cerebellums of the respective mice rapidly removed and transferred to ice-cold cutting buffer. The cerebellum was fixed to the pedestal of a Vibroslicer™ (Campden Instruments Ltd., Loughborough, England) with cyanoacrylate. Finally, the slices were fixed in 4% paraformaldehyde. The slices provided were 100µm thick sagittal sections through the vermis, along with other sections within the cerebellar hemispheres. In total, there were eight samples - four were mdx mice and the other four were control mice.

Immunohistochemistry

The cerebellar slices were stained with Calbindin due to its superiority to Nissl Staining and facilitation of cerebellar PC counting. [6,7] All vermal slices selected were stained with Calbindin and immunohistochemistry was performed under the laboratory standard protocol for immunohistochemistry. [8]

Cell counts

PCs from every section were counted with the Stereo Investigator® (MicroBrightField, Inc., Williston, USA) in the Prince of Wales Medical Research Institute, Sydney, Australia (POWMRI). The counting technique potentially overestimated PC numbers. However, given that the same method was applied to every section analysed, it was decided not to use any correction factor.

The PC number was collected from the three separate sections of the vermal slice: from lobe 10; lobes 3 and 4/5 from the anterior cerebellum and lobes 6 and 8 from the posterior cerebellum.

Although the ideal thickness of slices for the purpose of counting PC numbers is 50 µm, the slices in this study were 100 µm thick. Therefore, three cell counting techniques were used to estimate PC numbers.

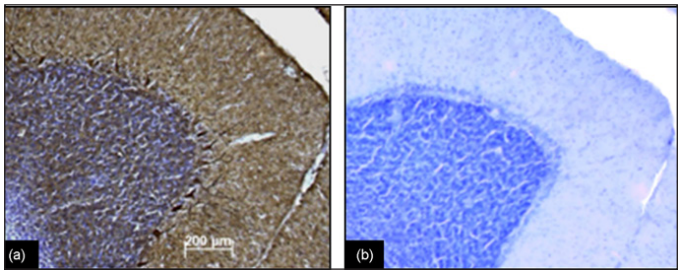

Figure 1. *Comparison of Nissl stained and Calbindin-D28k immunostained sections. (a) Calbindin immunopositive PCs clearly visible along the PC layer. (b) PCs not visible in Nissl stained section. [7]*

- Method 1: Actual number of PCs per lobe (actual PC count without mathematical adjustment).
- Method 2: The randomised estimated counts. Using the Stereo Investigator®, five sections were randomly selected along the PC layer of variable distances and the number of PCs within those sections counted. Results were averaged. The total possible PC count was estimated by multiplying that average with the total length of the PC layer in that lobe.
- Method 3: The estimated maximum possible count. Three segments of variable length along the PC layer with the densest population of PCs were selected. The number of PCs per unit length was averaged and multiplied by the total length of the PC layer of that lobe.

Statistical Methods

The unpaired t-test was applied to test the equivalence of PC counts between mutant and normal mice at different lobules statistically. Where the number of PCs was normally distributed the Mann-Whitney U test was used. Box-and-whisker plots were drawn to illustrate a comparison of the PC numbers in the mutant and normal mice. All tests were performed using STATA™ 9.0 (Stata Corporation, College Station, TX, USA). All statistical evaluations were made assuming a two-sided test with significance level of 0.05.

Results

Qualitative assessment

On examination of the slides without microscopy, there were no obvious differences between the mdx from the normal mice. The calbindin staining appeared homogenously distributed. It must be noted however that these missing rows may be a natural occurrence in the groups of mice tested.

Statistical assessment

The unpaired Student t-test (Table 1) and the Mann-Whitney U test (Table 2) did not reveal any significant difference in the number of PC counts between mutants and controls at different lobules, regardless of the method of cell counting. It is likely, however, that our small sample size precludes statistical assessment using these tests.

Actual PC count and box-and-whisker plots

Box-and-whisker plots demonstrate a difference in the median PC count at lobules 3, 4/5 and 6 between mutant and normal mice. The findings of our PC counts are presented below, organised by the method of cell counting used.

Table 1. *P-values of the comparison of the number of PC counts between mutant mice and controls using unpaired t-test.*

Site	Method 1	Method 2	Method 3
Lobule 10	0.2608	0.6899	0.7885
Lobule 8	0.7976	0.5258	0.6430
Lobule 6	0.0980	0.2294	0.9254
Lobule 4/5	0.0924	0.1671	0.3774
Lobule 3	0.1455	0.3410	0.2890

Table 2. *P-values of the comparison of the number of PC counts between mutant mice and controls using Mann-Whitney U test.*

Site	Method 1	Method 2	Method 3
Lobule 10	0.2482	0.7728	1.0000
Lobule 8	0.8845	0.2454	0.7728
Lobule 6	0.1489	0.1489	0.7728
Lobule 4/5	0.0833	0.1489	0.2482
Lobule 3	0.2482	0.4678	0.2482

Method 1

Our data showed significant differences in the median PC count between the mutant and control groups (Figure 2). Differences were most apparent in lobules 3, 4&5, 6 and 10. The largest difference was found in lobules 4&5 and 6.

Figure 3 demonstrates the mean PC number in each group. This figure illustrates a decrease in the mean PC number in lobules 3, 4/5 and 6. The percent decrease in the mdx group was 33.5%, 39% and 33% in lobules 3, 4/5 and 6 respectively.

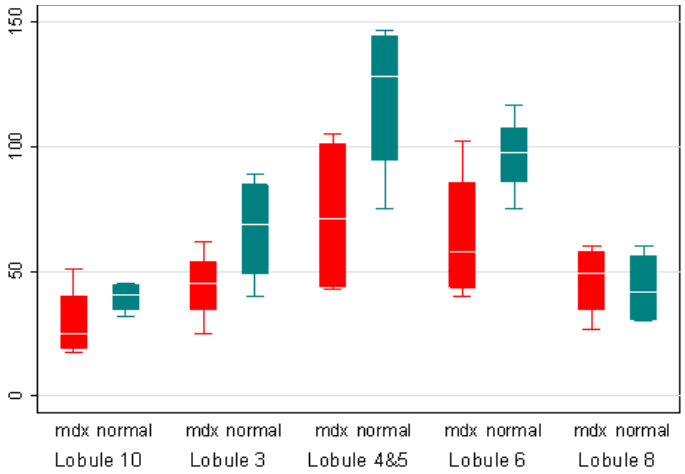

Figure 2. *Box-and-whisker plot of PC counts in mutants and controls at different lobules using method 1.*

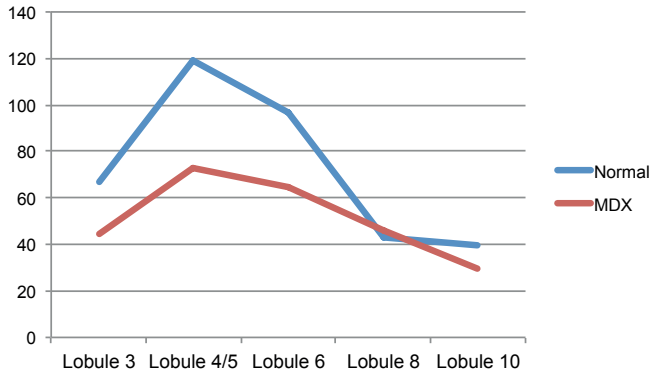

Figure 3. *Method 1 – Actual PC count. x-axis: lobule number; y-axis: the mean results of the PC number in the mdx and normal mice.*

Table 3. *Method 1 - table of results of the actual PC count.*

	Mouse	Lobule 10	Lobule 8	Lobule 6	Lobule 4/5	Lobule 3
MDX	1	51	60	102.5	105	62
	2	29	56	69	97.5	44.5
	3	17.5	42.5	47	45	25
	4	20.5	26.5	40	43	45.5
Control	1	44	52.5	97	146.5	89
	2	32	30	75	114	80.5
	3	37.5	31	117	75	40
	4	45	60	98.5	142	57.5

Method 2

Figures 4 and 5 demonstrate large differences in the number of PCs in lobules 3, 4/5 and 6. The most significant difference was found in lobule 4/5. The mean PC count in the mutant group was decreased by 16%, 30% and 24.5% in lobules 3, 4/5 and 6 respectively.

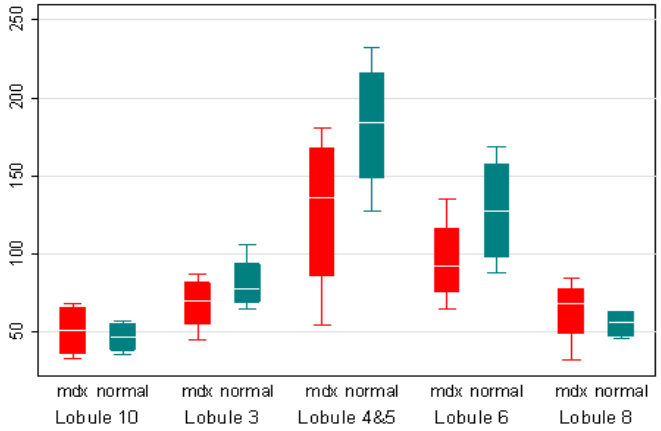

Figure 4. *Box-and-whisker plot of PC counts in mutants and controls at different lobules using method 2.*

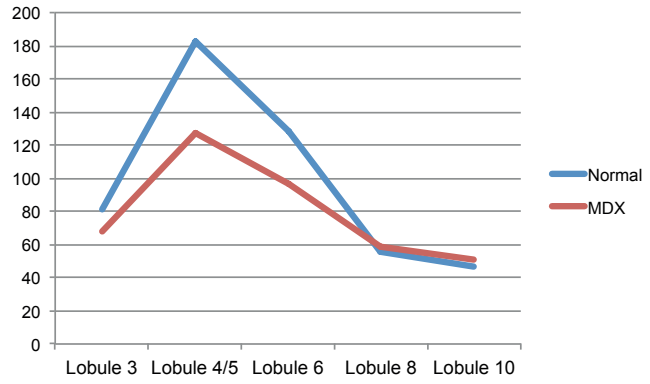

Figure 5. *Method 2 - The estimated randomised PC counts. x-axis: lobule number; y-axis: the mean results of the PC number in the mdx and normal mice.*

Table 4. *Method 2 - table of results of the estimated randomised PC counts.*

	Mouse	Lobule 10	Lobule 8	Lobule 6	Lobule 4/5	Lobule 3
MDX	1	68	65	135.6	180.9	87.6
	2	63.7	71	97.8	154.7	75.8
	3	38.9	65.8	86.7	116.8	65
	4	33.1	32.4	65	55	44.8
Control	1	57	63.5	107.4	199.2	106.4
	2	35.6	45.8	88	170	81
	3	41	48.8	169	127.5	65
	4	53.2	63.5	147	232	73.5

Method 3

Figures 6 and 7 demonstrate differences in the PC number, particularly in lobules 3 and 4/5. The most significant difference was found in lobule 4/5. However, this difference was less significant than those observed in method 1 and 2. Figure 7 illustrates a decrease in PC number in the mutant group by 16% and 16% in lobules 3 and 4&5 respectively.

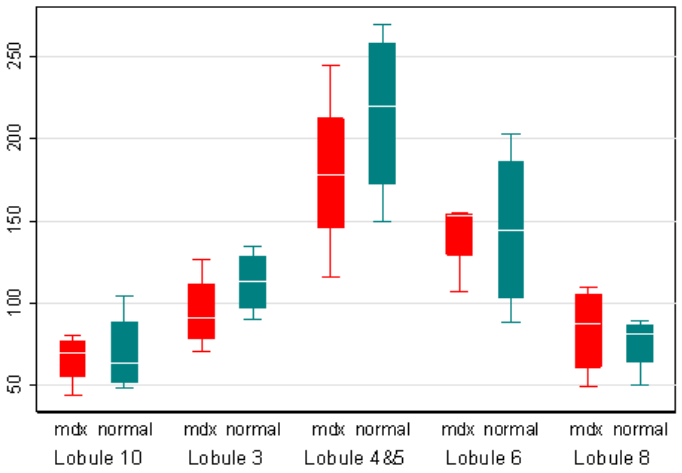

Figure 6. *Box-and-whisker plot of PC counts in mutants and controls at different lobules using method 3.*

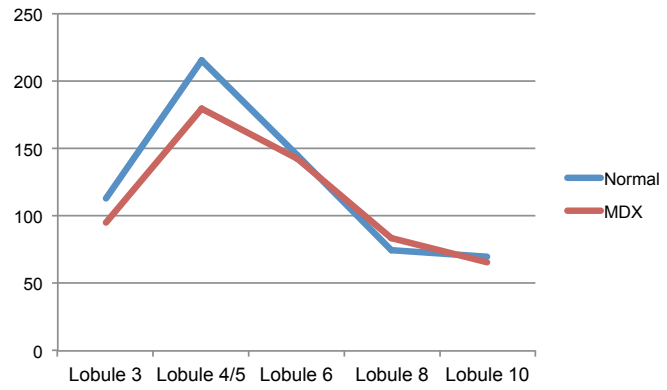

Figure 7. *Method 3 – The estimated maximum possible PC count. x-axis: lobule number; y-axis: the mean results of the PC number in the mdx and normal mice.*

Table 5. *Method 3 - tabulated results of the estimated maximum possible PC count.*

	Mouse	Lobule 10	Lobule 8	Lobule 6	Lobule 4/5	Lobule 3
MDX	1	80	101	151.7	244.9	96.7
	2	73	73	155.4	175.9	126.5
	3	65.7	110	106.8	180.6	71
	4	44.2	48.8	153.69	115.6	85.4
Control	1	72.5	78	118.5	246	123
	2	47.9	49.7	88	194.5	103.6
	3	55	89	203	150	90
	4	104	84	169.1	270	134.7

Sample size estimation

Table 6 summarises the mean and standard deviation (S.D.) of the number of PC counts among each group of mice at different lobules. Sample size estimation was based on these results. Considering multiple tests by adjustment using the Bonferroni method, two-sided unpaired t-test using equal variance shows that the sample sizes

Table 6. *Summary of the number of PC counts between mutant mice and controls at different lobules.*

	Method 1 (mean ± S.D)		Method 2 (mean ± S.D)		Method 3 (mean ± S.D)	
Site	Control	Mutant	Control	Mutant	Control	Mutant
Lobule 10	39.6±6.1	29.5±15.1	46.7±10.1	50.9±17.5	69.9±25.0	65.7±15.5
Lobule 8	43.4±15.2	46.3±15.2	55.4±9.4	63.6±22.3	75.2±17.6	83.2±27.8
Lobule 6	96.9±17.2	64.6±28.1	127.9±36.8	96.3±29.5	144.7±51.3	141.9±23.4
Lobule 4/5	119.4±32.9	72.6±33.2	182.2±44.4	126.9±54.7	215.1±53.6	179.3±52.8
Lobule 3	66.8±22.3	44.3±15.1	81.5±17.9	68.3±18.2	112.8±19.9	94.9±23.5

required to compare the PC counts between mutant mice and controls at significance level of 0.05 and power of 0.8 using method 1, 2 and 3 would be 66 (33 control and 33 mutant), 546 (273 control and 272 mutant), and 9,490 (4,745 mutant and 4,745 control), respectively. The number of mice needed for this study would be 11,610 (5,805 control and 5,805 mutant) to test the PC counts between mutant mice and controls at different lobules using different counting methods at a significance level of 0.05 (the significance level should be 0.05/15 after adjustment if three methods are used in the same study) and power of 0.8.

Discussion

This study found limited evidence suggestive of a reduction in PC number in the mdx mouse, particularly in lobules 4/5 and 6. Several previous studies have also analysed PC numbers and attempted to correlate PC number with other mutations, such as the Staggerer, Lurcher chimera, Stumbler, Reeler and Weaver mice. [9-13]

This analysis has yielded statistically insignificant results for all counting methods and for every lobule. However, it is likely that the small sample size precludes statistical analysis. Thus, data is presented via box-and-whisker plots and the tabulation of raw data. Differences between the mutant and control group are demonstrated, particularly in lobules 4/5 and 6 across all three methods.

The number of PCs is influenced by intrinsic mechanisms (such as the organism's genetic makeup) and extrinsic mechanisms (for example, hormonal factors which determine stem cell survival and recruitment).

PCs in lobules 1 and 10 are the earliest to mature, whereas those in lobules 6, 7 and 8, that is, lobules in the posterior cerebellum, are the latest. [14] The results in method 1 demonstrate a marked decrease in the PC population in lobules 3, 4/5, a marginal decrease in lobules 6 and 10 and a negligible decrease in lobule 8. Therefore, it is suggested that since the lobules which are affected tend to be those which mature earlier, the stage at which DMD affects the cerebellum and notably the PCs may be between the neurogenesis of PCs and the full maturation of all the lobules.

Furthermore, other neurological structures have been shown to be affected by a lack of dystrophin. For example, in the peripheral nervous system, utrophin and the short dystrophin soform (Dp116) are co-localised at the outermost layer of the myelin sheath of nerve fibers. Dystrophin is also present in the outer plexiform layer in the retina and cochlear hair cells, and is mostly absent from subcortical neurons. [15,16] Thus, a lack of dystrophin has shown to cause sensorineural hearing loss in mdx mice. Given that neurological structures are affected by a lack of dystrophin, we postulate that the lack of dystrophin may have an effect on cerebellar PCs.

Moreover, dystrophin has also been shown to regulate Ca^{2+} flux and its absence leads to increased intracellular calcium. [17,18] Raynor and Mulroy [15] have suggested that dystrophin may play a role in protecting the cell membrane of cochlear hair cells against mechanically induced damage, by regulation of calcium influx or stabilisation of membrane structure as previously described. Given that PCs begin to express calcium-binding protein or calbindin as they mature, [5] we speculate that perhaps this dysregulation of Ca^{2+} flux due to a lack of dystrophin may be responsible for potential problems with maturation at this stage.

Conclusion

It was Duchenne himself who first noted cognitive deficits in patients with DMD. [19] In addition to other morbidities, DMD has since been shown to cause a reduction in the IQ score of boys with the disease. IQ scores in this group are 1 standard deviation lower than the average population. [2] Clinically, our study examines the effect of DMD on the cerebellar PC population and the cells associated with it. Our study findings suggest a trend towards a decrease in the number of PCs in mdx mice. Although we are unable to pin-point the exact pathophysiology underlying the intellectual deficits in those with DMD, we propose several points in the neurogenesis of PCs where DMD may disrupt PC development. Our study findings suggest the need for a large trial to determine the statistical significance of differences in PC numbers in mdx and normal mice.

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Conflicts of Interest

None declared.

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References

[1] Dubowitz V. Muscle disorders in childhood, Second edition. Philadelphia, PA. WB Saunders, 1995.
[2] Anderson JE, Head SI, Rae C, Morley JW. Brain function in Duchenne muscular dystrophy. Brain 2002;125(1):4-13.
[3] Blake DJ, Kroger S. The neurobiology of Duchenne muscular dystrophy: Learning lessons from muscle? Trends Neurosci 2000;23(3):92-9.
[4] Brodal A. Neurological anatomy in relation to clinical medicine, Third edition. New York. Oxford University Press, 1981.
[5] Wang VY, Zoghbi HY. Genetic regulation of cerebellar development. Neuroscience 2001;2(7):484-91.
[6] Nakagawa S, Watanabe M, Isobe T, Kondo H, Inoue Y. Cytological compartmentalisation in the staggerer cerebellum, as revealed by calbindin immunohistochemistry for Purkinje cells. J Comp Neurol 1998; 395(1):112-20.
[7] Whitney ER, Kemper TL, Rosene DL, Bauman ML, Blatt GJ. Calbindin-D28k is a more reliable marker of human Purkinje cells than standard Nissl stains: a stereological experiment. J Neurosci Methods 2008;168(1):42-7.
[8] Paxinos G, Watson C, Carrive P, Kirkcaldie M, Ashwell K. Chemoarchitectonic atlas of the rat brain, Second edition. San Diego, CA. Elsevier Academic Press, 2009.
[9] Hadj-Sahraoui N, Frederic F, Zanjani H, Delhaye-Bouchaud N, Herrup K, Mariani J. Progressive atrophy of cerebellar Purkinje cell dendrites during aging of the heterozygous staggerer mouse (Rora(+/sg)). Dev Brain Res 2001;126(2):201-9.
[10] Maricich SM, Soha J, Trenkner E, Herrup K. Large neuron death and migration deficits in weaver. J. Neurosci 1997 17(10):3675–83.
[11] Herrup K, Shojaeian-Zanjani H, Panzini L, Sunter K, Mariani J. The numerical matching of source and target populations in the CNS: The inferior olive to purkinje cell projection. Dev Brain Res 1996;96(1):28-35.
[12] Heckroth JA, Goldowitz D, Eisenman LM. Purkinje cell reduction in the reeler mutant mouse: a quantitative immunohistochemical study. J Comp Neurol 1989;279(4):546-55.
[13] Caddy KW, Sidman RL. Purkinje cells and granule cells in the cerebellum of the stumbler mutant mouse. Dev Brain Res 1981;227(2):221-36.
[14] Altman J. Postnatal development of the cerebellar cortex in the rat II. Phases in the maturation of Purkinje cells and of the molecular layer. J Comp Neurol 1972;145(4):399–463.
[15] Lidov HG, Byers TJ, Kunkel LM. The distribution of dystrophin in the murine central nervous system: an immunocytochemical study. Neuroscience 1993;54(1):167-87.
[16] Raynor EM, Mulroy MJ. Sensorineural hearing loss in the mdx mouse: A model of Duchenne muscular dystrophy. Laryngoscope 1997;107(8):1053-6.
[17] Clarke MSF, Khakee R, McNeil PL. Loss of cytoplasmic basic fibroblast growth factor from physiologically wounded myofibers of normal and dystrophic muscle. J Cell Sci 1993;106(1):121-33.
[18] Fong P, Turner PR, Denetclaw WF, Steinhardt RA. Increased activity of calcium leak channels in myotubes of Duchenne human and mdx mouse origin. Science 1990;250(4981):673-5.
[19] Duchenne GBA. Recherches sur la paralysie musculaire pseudohypertrophique, ou paralysie myo-sclerosique. Arch Gen Med 1861;11:5-25.

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