UNIVERSITI PUTRA MALAYSIA

METABOLIC AND FUNCTIONAL CHARACTERISATION OF ADULT SKELETAL MUSCLE IN DOWN SYNDROME MOUSE MODEL (Ts1CJe) FOR INSIGHTS INTO HYPOTONIA IN HUMAN CONDITION

LIM CHAI LING

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By

LIM CHAI LING

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

April 2017

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

METABOLIC AND FUNCTIONAL CHARACTERISATION OF ADULT SKELETAL MUSCLE IN DOWN SYNDROME MOUSE MODEL (Ts1Cje) FOR INSIGHTS INTO HYPOTONIA IN HUMAN CONDITION

By

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April 2017

Chairman : Cheah Pike See, PhD
Faculty : Medicine and Health Sciences

Down syndrome (DS) is a genetic condition resulting from a partial or full triplication of human chromosome 21. In addition to intellectual disability, DS is frequently associated with hypotonia. However, little is known about its underlying mechanism. In this study, the trisomic Ts1Cje mouse, a DS murine model, was employed to explore the possible mechanisms of DS-associated hypotonia. The hypotheses of this study are the over dosage of trisomic genes disrupts the population size and the cellular functionality of trisomic Ts1Cje satellite cells, as well as, the metabolic pathways in trisomic Ts1Cje skeletal muscle. Eventually, they lead to hypotonia seen in DS. In order to determine the satellite cell population in trisomic Ts1Cje skeletal muscle, myofibres derived from the EDL of the adult trisomic Ts1Cje mice and its age-matched disomic wild-type control littermates were isolated. The associated satellite cells were then quantified by using immunostaining for Pax7 (a marker for quiescent satellite cells). The results showed no significant variation in terms of the satellite cell populations between the two genotypes, indicating that the depletion of satellite cell populations may not a primary cause of DS-associated hypotonia. Additionally, the average number of myonuclei present in each EDL myofibre of the trisomic Ts1Cje mice was also investigated. The data obtained suggest that there was no significant difference in the average number of myonuclei per myofibre genotypes between the two genotypes. This finding suggested the trisomic Ts1Cje myofibres are normal in size. Meanwhile, the intrinsic cellular functionality of satellite cells between the two genotypes was also determined. Satellite cells derived from the EDL of the two genotypes were isolated and cultured in high-serum containing conditioned medium. Subsequently, the *in vitro* self-renewal, proliferative and differentiation activity of these myogenic precursor cells were assessed at 24, 48 and 72 h after cell seeding. These progenies were distinguished on the basis of Pax7 and MyoD (a marker for activating satellite cells) expression patterns. Furthermore, the results (proliferation and differentiation potential) obtained were later validated using Ki67 (a marker for proliferating cells)

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and MyoD expression patterns. These findings demonstrated that there was no difference between the satellite cells of the two genotypes in their ability to self-renew, proliferate and differentiate, indicating that alteration of the cellular function of satellite cells is not a primary cause of DS-associated hypotonia. Additionally, the metabolic profiles of trisomic Ts1Cje skeletal muscle were also evaluated using a non targeted metabolomics strategy. The hydrophilic and hydrophobic metabolites present in *gastrocnemius* (GA) samples of the two genotypes were extracted using methanol/chloroform/water partitioning-based protocol and subsequently were characterised by using ^{1}H NMR spectroscopy combined with multivariate data analysis. The findings revealed that guanidinoacetate, histidine, adenosine mono-phosphate and glutamine were found to be at lower levels in the trisomic Ts1Cje skeletal muscle, indicating that alteration of energy, glutamate and histidine pathway metabolism in trisomic Ts1Cje skeletal muscle may underlie the hypotonia seen in DS. In conclusion, the perturbation of metabolic profile resulted from the over dosage of trisomic genes is the primary cause of DS-associated hypotonia.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

PENCIRIAN METABOLIK DAN FUNGSI SELULAR OTOT RANGKA MENCIT SINDROM DOWN (Ts1Cje) BAGI SIASATAN PUNCA YANG MENYEBABKAN KELEMAHAN OTOT DALAM KALANGAN PESAKIT SINDROM DOWN

Oleh

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April 2017

Pengerusi : Cheah Pike See, PhD
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Sindrom Down (DS) adalah satu keadaan genetik yang terhasil daripada penigaan sebahagian atau penuh kromosom manusia 21. Selain daripada kecacatan intelektual, individu DS juga sering dilapor dengan hipotonia. Walau bagaimanapun, mekanisme yang menyebabkan sindrome tersebut masih belum dikenalpastikan. Dalam kajian ini, kami menggunakan mencit trisomik Ts1Cje, satu jenis model mencit DS, untuk meneroka mekanisme yang menyebabkan hipotonia berkaitan dengan DS. Hipotesis dalam kajian ini adalah bahawa kelebihan dos gen trisomi akan mengganggu jumlah populasi dan fungsi selular sel-sel satelit, serta akan mengusikan laluan metabolik dalam otot rangka mencit Ts1Cje. Akhirnya, sebab-sebab tersebut akan menyebabkan hipotonia yang sering dijumpa di kalangan pesakit DS. Dalam usaha untuk mencirikan jumlah populasi sel satelit, serat otot rangka *extensor digitorum longus* (EDL) telah dikutip daripada mencit dewasa trisomik Ts1Cje dan mencit kawalan yang sepadan umur. Sel-sel satelit pada setiap serat otot telah dikaji dengan Pax7 melalui immunohistokimia (penanda spesifik bagi sel satelit). Keputusan eksperimen ini telah menunjukkan tiada perbezaan secara signifikan dari segi populasi sel satelit antara kedua-dua jenis genotip, mencadangkan bilangan dalam populasi sel satelit ini tidak menyumbang kepada hipotonia berkaitan dengan DS. Selain itu, purata mionukleus dalam setiap serat otot berasal dari otot EDL juga disiasat Keputusan eksperimen ini menunjukkan tiada perbezaan yang ketara dalam bilangan purata mionukleus antara otot rangka mencit Ts1Cje dan mencit kawalan untuk kedua-dua jantina. Keputusan ini membuktikan bahawa mencit Ts1Cje mempunyai saiz serat otot yang normal. Fungsi selular intrinsik sel-sel satelit antara kedua-dua genotip juga disiasat. Dalam eksperimen ini, sel-sel satelit yang berasal dari otot EDL mencit trisomik Ts1Cje jantan dewasa dan mencit kawalan yang sepadan umur telah dikulturkan dalam medium yang kaya dengan serum. Seterusnya, aktiviti sel-sel satelik seperti pembaharuan diri *in vitro*, proliferatif dan diferensiasi telah dinilaikan pada 24, 48 dan 72 jam dalam keadaan kultur. Progeni yang berikut

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telah dibezakan berdasarkan perwarnaan immunohistokimia dengan menggunakan Pax7 dan MyoD. Tambahan pula, keputusan eksperimen (proliferatif dan diferensiasi) juga telah disahkan melalui perwarnaan immunohistokimia dengan menggunakan Ki67 dan MyoD. Keputusan kajian ini telah menunjukkan bahawa tiada perbezaan secara signifikan antara sel-sel satelit bagi kedua-dua genotip dari segi keupayaan untuk memperbaharui diri, pertumbuhan dan diferensiasi. Hasil kajian ini telah mencadangkan bahawa fungsi selular sel satelit tidak memainkan peranan dalam menyebabkan hipotonia yang berkaitan dengan DS. Di samping itu, profil metabolik otot rangka mencit Ts1Cje juga diperiksa dengan menggunakan strategi metabolomiks yang bersasaran bebas. Metabolit hidrofilik dan hidrofobik dari otot rangka *gastrocnemius* (GA) telah diekstrak dengan menggunakan protokol ekstrak metanol/ kloroform/ air dan seterusnya telah diciri dengan menggunakan ^{1}H NMR spektroskopi bergabung dengan kaedah "*multivariate data analysis*". Keputusan eksperimen ini menunjukkan bahawa kandungan guanidinoacetat, histidin, adenosin mono-fosfat dan glutamin dalam otot rangka mencit trisomik Ts1Cje adalah lebih rendah secara signifikan berbanding dengan mencit kawalan. Keputusan ini mencadangkan bahawa laluan metabolisme tenaga, laluan metabolism glutamat dan laluan metabolisme histidin memainkan peranan yang penting dalam menyebabkan hipotonia yang berkaitan dengan DS. Kesimpulannya, sel-sel satelit dari otot rangka memainkan peranan yang minimum dalam menyebabkan hipotonia. Walabagaimanapun, kelebihan dos gen trisomi telah mengakibatkan gangguan laluan metabolik dan seterusnya mengakibatkan hipotonia yang berkaitan dengan DS.

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LIST OF ABBREVIATIONS

1D	1-dimensional
ADP	Adenosine diphosphate
AGAT	L-arginine: glycine amidinotransferase
AK	Adenylate kinase
AMP	Adenosine monophosphate
ANT	Adenine nucleotide translocator
ASD	Autism spectrum disorder
ATP	Adenosine triphosphate
CO_2	Carbon dioxide
CSA	Cross sectional area
d	Doublet
D_2O	Deuterium oxide
DAPI	4, 6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DMD	Duchenne muscular dystrophies
DMEM	Dulbecco's Modified Eagle Medium
DS	Down syndrome
DSCR	DS critical region or chromosome region
ECM	Extracellular matrix
EDL	Extensor digitorium longus
FGF	Fibroblast Growth Factor
FID	Free induction decay
GA	Gastrocnemius
GAA	Guanidinoacetate
GAMT	N-guanidinoacetate methyltransferase
GC	Gas chromatography
H&E	Haematoxylin and eosin
HMDB	Human Metabolome Database
HPLC	High-performance liquid chromatography
HS	Horse serum
HSA21	Human chromosome 21
IACUC	Institute Animal Care and Use Committee
IL-8	Interleukin 8
m	Multiplet
mb	Multiplet broad
m/z	Mass/charge ratio
MMCD	Madison Metabolomics Consortium Database
MMU16	Mouse chromosome 16
MRF4	Myogenic regulatory factor 4
MRFs	Myogenic regulatory factors
MS	Mass Spectroscopy
Myf5	Myogenic factor 5
Myf6	Myogenic factor 6
MyoD	Myogenic determination factor 1
MVDA	Multivariate data analysis
NF-κB	Nuclear factor κB
NMR	Nuclear Magnetic Resonance

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OXPHOS	Oxidative phosphorylation
P	Postnatal
Pax	Paired box family of transcription factors
PAX	Paired box
PBS	Phosphate buffered saline
PCA	Principle component analysis
PFA	Paraformaldehyde
PLS-DA	Partial least squares-discriminant analysis
q	Quartet
RD	Recycle delay
ROS	Radical oxidative stress
s	Singlet
SAH	S-adenosyl homocysteine
SAM	S-adenosylmethionine
SEM	Standard error of the mean
Sod1	Superoxide dismutase 1
t	Triplet
UV	Unit variance
TA	Tibialis anterior
TAE	Tris-acetate-EDTA
TMS	Tetramethylsilane
TNF-α	Tumor necrosis factor alpha
TSP	3-(trimethylsilyl)propionic-2,2,3,3-d4 acid sodium salt
v/v	Volume/volume
w/v	Weight/volume
Zfp295	Zinc finger protein 295

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CHAPTER 1

INTRODUCTION

1.1 Background

Down syndrome (DS) is a genetic condition resulting from a partial or full triplication of human chromosome 21 (HSA21), which occurs at a rate of approximately 1 case per 700 live births worldwide (Roper and Reeves, 2006; Cdc.gov, 2016). The extra copy of chromosome 21 alters normal gene expression and eventually leads to a series of clinical manifestations affecting multiple organs. The clinical manifestations of DS are complex and have different degrees of penetrance and expressivity (Roper and Reeves, 2006). Some clinical manifestations, such as congenital cardiac diseases, thyroid disease and gastrointestinal disorders, affect only a subpopulation of DS individuals (Liu et al., 2014), whereas, certain clinical manifestations, such as intellectual disability, craniofacial abnormalities and hypotonia, are present in nearly all DS cases (Liu et al., 2014).

Hypotonia (muscle weakness) is a condition of low muscle tone, often accompanied by a slower speed of response together with a reduction in muscular endurance (Brault et al., 2015; Lisi and Cohn, 2011). It is cited as one of the most significant problems associated with DS. DS individuals have often been diagnosed with low muscular strength and delays in acquisition of fine and gross motor skills from early childhood. A previous study has reported that the force generated by the knee extensor muscle of DS individuals is approximately 40%-70% less than that generated by the knee extensor muscle of individuals with intellectual disabilities other than DS and those with normal intellectual ability (Cowley et al., 2012). This wide variation in muscle strength is comparable to the discrepancy observed among healthy young adults and elderly people. Unsurprinsingly, hypotonia will reduce the quality of life of DS patients. Unfortunately, the origin of DS-associated hypotonia is little known. Even though previous studies have suggested that DS-associated hypotonia could be due to premature aging and mitochondrial defects (Heffernan et al., 2009; Phillips et al., 2013; Brault et al., 2015). However, those findings are too superficial and scattered. Therefore, more extensive studies aiming to investigate the exact mechanism of DS-associated hypotonia are required in the coming days.

Due to ethical issues, there are arguments against using human subjects in basic research; therefore, a DS murine model, the trisomic Ts1Cje mouse, was employed as a tool in this study to unravel the causes of DS-associated hypotonia. Trisomic Ts1Cje was developed by Sago *et al.* in 1998 (Sago et al., 1998). This murine model carries a partial triplication of chromosome 16 spanning from the murine superoxide dismutase 1 (*Sod1*) gene to the Zinc finger protein 295 (*Zfp295*) gene. However, the *Sod1* gene is not functionally triplicated (Sago et al., 1998). Hence, it carries a normal copy number of the S*od1* gene (Olson et al., 2004). The trisomic Ts1Cje mouse is one of the commonly use murine models in DS research, because it displays

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a remarkable number of phenotypic characteristics reminiscent of those commonly observed among DS individuals. These phenotypic characteristics include structural, and cognitive alterations of the brain and craniofacial alterations (Liu et al., 2014). Thus, Ts1Cje mice have considerable value in the determining the mechanisms of DS-associated pathology. Moreover, in a study done by Bala (2016), trisomic Ts1Cje mice were also found to display reduced grip strength and locomotor activity as compared with their disomic wild-type control littermates (unpublished data; Appendix B). Thus, the trisomic Ts1Cje mouse is a suitable model to investigate the possible molecular and metabolic mechanisms of DS-associated hypotonia.

It is fairly well-accepted that over dosage of trisomic genes will disrupt the stability of the genome and eventually causes the perturbation of stem and progenitor cell growth. Various lines of evidence demonstrate that trisomic genes can affect the cellular activities of various types of stem/ progenitor cells, such as hematopoietic, neuronal and cardiac stem cells, either directly or by altering interactions with microenvironmental and temporal cues (De Vita et al., 2010; Roy et al., 2012; Bosman et al., 2015; Najas et al., 2015;), eventually causing both the dysmorphic features and pathogenesis of DS. However, whether a similar mechanism will apply to DS skeletal muscle is not yet known.

Satellite cells (also known as skeletal muscle resident cells) are rare mononuclear cells with low cytoplasmic content wedged between the basal lamina and sarcolemma of the postnatal skeletal muscle (Bischoff., 1990). In adult skeletal muscle, satellite cells are mitotically quiescent under normal circumstances. However, they are activated in response to exercise and muscle trauma (Boldrin, Muntoni and Morgan, 2010). Activated satellite cells will follow a well-characterised proliferation and differentiation program. Eventually, they will either fuse with each other or with the existing myofibres to generate new skeletal muscle tissue (Boldrin, Muntoni and Morgan, 2010). In addition to producing progeny destined for differentiation, a small population of satellite cells possess the ability to self-renew and thus, they are considered as reserve satellite cells (Sacco et al., 2013). These reserve satellite cells are crucial for the replenishment of the satellite cell pool. In summary, satellite cells play an essential role in skeletal muscle regeneration and the maintenance of skeletal muscle homeostasis (Tierney and Sacco, 2016). Therefore, a small defect in satellite cells can lead to a series of complications in recurrent regeneration. Many studies reported that a decrease in satellite cell populations contributes to a decrement in skeletal muscle functionality. For example, in the case of age-related sarcopenia, satellite cell populations in the skeletal muscle of elderly people are found to be lower as compared to the skeletal muscle from healthy young adults (Kadi et al., 2003; Shefer et al., 2006; Shefer et al, 2010). Nonetheless, the existing literature has not reported the influence of trisomic genes in the satellite cells of trisomic Ts1Cje skeletal muscle. Hence, the population size and cellular functionality of satellite cells in trisomic Ts1Cje skeletal muscle were assessed in this study. Additionally, the number of myonuclei was assessed to acquire supporting evidence for the results on satellite cell populations size and some additional information on myofibre size.

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On the other hand, previous literatures suggested that an overdose of trisomic genes would lead to the perturbation of metabolic pathways (Pogribna et al., 2001; Coppedè, 2009). A previous study also demonstrated that DS-associated hypotonia could be due to the disruption of metabolic pathways (Brault et al., 2015). However, those findings are too superficial and scattered. Therefore, a more extensive study aiming to extract more inclusive information on the metabolic profile of the trisomic Ts1Cje skeletal muscle is required. In this study, a ^{1}H NMR-based non-targeted metabolomics approach was employed.

Metabolomics is an emerging post-genomic field, tightly related to genomics and proteomics, which is concerned about the comprehensive identification and quantification of multiple small and low-molecular-weight metabolites (≤ 1500 Daltons) in biological samples (Gowda et al., 2008). The metabolome is the downstream product of the genome, transcriptome and proteome; hence, analysing the metabolome of a biological system could facilitate the extraction of an extensive and comprehensive description of pathway activity. Additionally, the total number of human metabolites (≈7,000) identified is comparatively modest as compared with genes (25,000), transcripts (100,000) and proteins (1,000,000) (Shah, Kraus and Newgard, 2012). Thus, interpreting metabolomics data will be relatively more proximal, simpler and less time consuming as compared with the interpretation of genomics and proteomics data.

With the availability of current advanced technologies, several hundred to thousands of small, low-molecular-weight molecules can be detected easily nowadays. Still, the detection sensitivity depends on the analytical platform. However, to date, there is no single technology able to capture the complete metabolome. Among the analytical platforms that can be utilised for metabolomics applications, mass spectroscopy (MS) and nuclear magnetic resonance (NMR) are the most commonly used techniques (Robertson and Lindon, 2005; Gowda et al., 2008; Nagrath et al., 2011). However, high-resolution ^{1}H NMR has been chosen for use in this study because it is the only technology capable of producing a comprehensive profile of metabolite signals without the need for preselection of measurement parameters or selection of separation or derivation procedures. Besides that, it also able to produce results that are highly reproducible as compared with MS (Robertson and Lindon, 2005; Gowda et al., 2008). Moreover, many recent studies demonstrated that ^{1}H NMR-based metabolomics have been used extensively to understand the pathogenesis of many diseases such as autism, cancer, cardiovascular diseases, stroke etc (Yap et al., 2010; Jung et al., 2011; Nagrath et al., 2011; Shah, Kraus and Newgard, 2012). Therefore, in this study, a non-targeted metabolomics strategy; combining ^{1}H NMR spectroscopy and multivariate data analysis was employed to obtain information on the metabolic profile of trisomic Ts1Cje skeletal muscle.

1.2 Problem statement

Proper motor skills are essentially important for a wide range of activities in our daily lives, from sitting and independent eating and drinking to walking and running.

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Delay in motor development due to hypotonia has considerable impacts on DS individuals' lives. It will not only interfere with their capability to perform daily living activities but will also limit their opportunities for independent living, vocational calling and economic independence. Ultimately, it leads to assisted living and lower quality of life among DS individuals. However, the origin of DS-associated hypotonia is less known. Therefore, an insight of the satellite cells and the metabolic profile in trisomic Ts1Cje skeletal muscle will have enormous implications for DS individuals' social and medical care needs.

1.3 Significance of the study

This study aims to provide fundamental knowledge of the underlying mechanism of DS-associated hypotonia. These findings will provide new clues to the etiology of DS-associated hypotonia and eventually, they will also give rise to better medical management of DS patients.

1.4 Hypotheses

The hypotheses of this study are the following:

1. Trisomic genes will cause a reduction of satellite cell populations in adult trisomic Ts1Cje skeletal muscle and eventually lead to DS-associated hypotonia.
2. Trisomic genes will affect the cellular capability of satellite cells in adult trisomic Ts1Cje skeletal muscle and eventually lead to DS-associated hypotonia.
3. Trisomic genes will cause a perturbation of metabolic pathways in the skeletal muscle of adult trisomic Ts1Cje mice and eventually lead to DS-associated hypotonia.

1.5 Objectives

1.5.1 General objective

This study seeks to investigate the effects of trisomic genes on satellite cells and on the alternation of the metabolic profile in trisomic Ts1Cje skeletal muscle, in order to provide insights into hypotonia seen among DS individuals.

1.5.2 Specific objectives

The specific objectives of this study are to determine the following:

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1. The effects of trisomic genes on satellite cell populations in trisomic Ts1Cje skeletal muscle;
2. The effects of trisomic genes on the cellular function of satellite cells in trisomic Ts1Cje skeletal muscle and
3. The effects of trisomic gene on the alteration of the metabolic profile in trisomic Ts1Cje skeletal muscle.

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