HISTOLOGICAL AND MOLECULAR CHARACTERISATION OF HYPOTONIA IN ADULT Ts1CJE MOUSE MODEL FOR DOWN SYNDROME

USMAN BALA

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By

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Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Doctor of Philosophy

HISTOLOGICAL AND MOLECULAR CHARACTERISATION OF HYPOTONIA IN ADULT Ts1CJE MOUSE MODEL FOR DOWN SYNDROME

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USMAN BALA

September 2016

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

Down syndrome (DS) is a genetic disorder caused by presence of extra copy of human chromosome 21 (Hsa21), a condition termed as trisomy 21. It is characterised by several number of clinical phenotypes such as intellectual disability, characteristic sets of facial features, cardiac defects and different systems anomalies. Motor dysfunction due to hypotonia is commonly seen in DS individuals and its etiology is yet unknown. Ts1Cje, which has a partial trisomy (*Mmu16)* synteny to Hsa21, is a mouse model for DS that is well reported to exhibit various typical neuropathologic features seen in DS individuals. However, hypotonia in Ts1Cje mouse has not been fully characterised. In this study, Ts1Cje mice was used to investigate the potential role of muscular and peripheral nervous systems defects in causing muscle weakness in DS individuals using molecular and histological approaches.

Behavioural assessment of the motor performance showed that, the forelimb grip strength (automated grip test) was significantly (P<0.0001) greater in the wild type mice compared to the Ts1Cje mice, regardless of gender. The average survival time of the wild type mice was significantly (P<0.01) greater compared to those of the Ts1Cje mice (P<0.01). In addition, the cumulative number of falls in the Ts1Cje mice was significantly (P<0.0001) greater than those of their wild type littermates. The wild type mice performed significantly (P<0.01) better than the Ts1Cje mice in the latency to maintain a coordinated motor movement against the rotating rod (accelerated speed).

Relative expression of both trisomic (*Itsn1*, *Syjn1* and *Rcan1*) and the non-trisomic genes (*Lamc1*, *Leprel1*, *Myl6b*, *Msn* and *Pgm5)* showed no significant difference in both quadriceps and triceps of Ts1Cje mice as compared with the wild type. Expression of Myf5 was significantly (P<0.05**)** reduced in triceps of Ts1Cje mice while MyoD expression was significantly (P<0.05) increased in quadriceps of Ts1Cje mice as compared with wild type.

Morphological evaluation of the skeletal muscles revealed no pathological changes in Ts1Cje mice. Analysis of both the ATPase-stained and NADH diaphorase-stained sections showed a significantly (P<0.001) higher population of type I fibres both in quadriceps and triceps of wild type than that of Ts1Cje mice. There was significantly (P<0.01) higher population of the COX deficient fibres in quadriceps and triceps of Ts1Cje mice as compared with the wild type.

An ultrastructural assessment of nerve fibres revealed no morphological differences between the Ts1Cje and wild type mice. The g ratio of the Ts1Cje mice was significantly (P<0.0001) greater compared to the wild type mice. The myeline thickness was significantly (P<0.0001) thinner in nerve fibres of the Ts1Cje mice as compared to that of the wild type mice. Both adult and aging groups of the Ts1Cje mice further exhibited significantly (P<0.001) lower conduction velocity compared with their aged matched wild types.

Ts1Cje mice exhibited weaker muscle strength. The lower population of the type I fibres and COX deficient fibres together with the decreased level of myeline in Ts1Cje mice may contribute to the muscle weakness seen in this mouse model for DS.

Key words: Down syndrome; muscle weakness; Ts1Cje mice; skeletal muscle; peripheral nervous system

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia
sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENCIRIAN HIPOTONIA DARI SEGI SELULAR DAN MOLEKULAR PADA MODEL SINDROM DOWN TIKUS DEWASA Ts1CJE

Oleh

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September 2016

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Sindrom down (DS), juga dikenali sebagai Trisomi 21, merupakan kromosom tidak normal yang disebabkan oleh kelebihan pada kromosom manusia 21 (Hsa21). Sindrom ini menunjukkan beberapa ciri fenotip klinikal seperti kecacatan akal, ciri-ciri struktur muka, kecacatan jantung dan pelbagaiabnormaliti pada sistem badan.. Sistem motor terganggu yang disebabkan oleh hipotonia sering dikaitkan dengan individu DS dan etiologinya masih tidak diketahui. Ts1Cje merupakan model tikus DS yang mempunyai separuh trisomi (*Mmu16*) berkaitan dengan Hsa21. Ia mempamerkan pelbagai ciri neuropatologi yang sering dikaitkan dengan individu DS. Walaubagaimanapun, hipotonia pada tikus Ts1Cje masih belum dicirikan sepenuhnya. Pada kajian ini, tikus Ts1Cje telah digunakan untuk mengkaji peranan kecacatan sistem otot dan saraf periferi yang mengakibatkan kelemahan otot pada individu DS dengan menggunakan pendekatan molekular dan selular.

Penilaian tingkah laku bagi prestasi motor menunjukkan bahawa, kekuatan cengkaman anggota hadapan (ujian cengkaman automatik) adalah lebih tinggi secara signifikan (P<0.0001) pada tikus kawalan berbanding dengan tikus Ts1Cje, tanpa mengira jantina. Tikus kawalan mempunyai skor yang lebih tinggi secara signifikan pada ujian wayar tergantung berbanding dengan tikus Ts1Cje (P<0.01). Di samping itu, jumlah relatif jatuh pada kumpulan tikus Ts1Cje adalah lebih tinggi secara signifikan (P<0.0001) berbanding dengan tikus kawalan. Tikus kawalan dapat mengawal pergerakan motor pada rod berputar (fasa dipercepatkan) dengan lebih baik secara signifikan (P<0.01) berbanding dengan tikus Ts1Cje.

Untuk kedua-dua otot kuadriseps dan triseps tikus Ts1Cje, analisis relatif ekspresi bagi lima gen yang bukan trisomi (*Lamc1, Leprel1, Myl6b, Msn* dan *Pgm5*) dan tiga gen trisomi (*Itsn1, Syjn1* dan *Rcan1*) telah menunjukkan bahawa tiada perbezaan yang signifikan berbanding dengan tikus kawalan. . Ekspresi protein Myf5 telah menunjukkan pengurangan secara signifikan (P<0.05) di triseps tikus Ts1Cje berbanding dengan tikus kawalan manakala eksperesi protein MyoD menunjukkan peningkatan secara signifikan di kuadriseps tikus Ts1Cje berbanding dengan tikus kawalan.

Penilaian morfologi otot rangka menunjukan tiada sebarang perubahan patologi pada tikus Ts1Cje. Analisi pewarnaan ATPase dan NADH diaphorase menunjukkan populasi gentian jenis I adalah lebih banyak secara signifikan (P <0.001) di kedua-dua kuadriseps dan triseps pada tikus kawalan, berbanding dengan tikus Ts1Cje. Untuk kedua-dua genotip yang dikaji, pewarnaan COX telah menunjukkan pengurangan yang signifikan (P<0.01) untuk gentian COX-positif di kuadriseps dan triseps tikus Ts1Cje berbanding dengan tikus kawalan.

Penilaian ultrastruktur bagi gentian saraf menunjukan tiada sebarang perbezaan morfologi di antara tikus Ts1Cje dan tikus kawalan. Tikus Ts1Cje telah menunjukkan nisbah g yang lebih tinggi secara signifikan (P<0.0001) berbanding dengan tikus kawalan. Manakala, ketebalan meilin di dalam gentian saraf tikus Ts1Cje adalah lebih nipis secara signifikan (P<0.0001) berbanding dengan tikus kawalan. Kedua-dua kumpulan tikus Ts1Cje dewasa dan tua telah menunjukkan saraf pengaliran halaju yang lebih rendah secara signifikan (P<0.001) berbanding dengan tikus kawalan.

Tikus Ts1Cje menunjukan kekuatan otot yang lemah. Perbezaan di dalam gentian jenis I dan kekurangan gentian COX serta kekurangan meilin pada tikus Ts1Cje boleh menyumbangkan kepada kelemahan otot yang dapat diperhatikan pada model tikus sindrom Down ini.

Key words: sindrom Down; kelemahan otot; tikus Ts1Cje; otot rangka; sistem saraf periferi

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This thesis was submitted to the Senate of the Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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Declaration by Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of this thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) were adhered to.

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LIST OF ABBREVIATIONS

ACh	Acetylcholine
IACUC	Institutional Animal Care and Use Committee
App	Amyloid precursor protein
APS	Ammonium persulfate
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
bp	Base pair
BSA	Bovine serum albumin
Ca^{2+}	Calcium ion
Cbr1	Carbonyl reductase 1
CCD	Charge coupled device
cDNA	Complementary deoxyribonucleic acid
CMAP	Compound muscle action potential
CNS	Central nervous system
COX	Cytochrome C oxidase
Cp	Crossing point
dap160	Dynamin associated protein 160
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide triphosphate
DPX	Distyrene plasticizer xylene
DS	Down syndrome
DSCR	Down syndrome critical region
DSCR1	Down syndrome critical region 1

DTT	Dithiothreitol
Dyrk1a	Dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A
EDTA	Ethylenediaminetetraacetic acid
ERM	Ezrin, radixin & moesin
FC	Fold change
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Guanine-cytosine
gDNA	Genomic deoxyribonucleic acid
Grik1	Glutamate receptor, ionotropic, kainate 1
GRMRC	Genetics and Regenerative Medicine Research Centre
H & E	Haematoxylin and eosin
HCl	Hydrochloric acid
HKG	Housekeeping genes
Hmbs	Hydroxymethylbilane synthase
HRP	Horse-peroxidase
Hsa21	*Homo sapiens* autosome 21 (Human chromosome 21)
Ifngr2	Interferon gamma receptor 2
Itsn1	Intersectin1
K^+	Potassium ion
L3	Lumbre 3
L4	Lumbre 4
L5	Lumbre 5
Lamc1	Laminin gamma 1
Leprel1	Leprecan-like 1
Mmu10	Mouse chromosome 10

Mmu12	Mouse chromosome 12
Mmu16	Mouse chromosome 16
Mmu17	Mouse chromosome 17
MPCs	Muscle progenitor cells
MRF4	Muscle-specific regulatory factor 4
MRFs	Myogenic regulatory factors
mRNA	Messenger ribonucleic acid
Mrpl39	Mitochondrial ribosomal protein L39
Msn	Moesin
Myf5	Myogenic factor 5
Myl6b	Myosin light polypeptide 6B
MyoD	Myogenic determination factor 1
Na^+	Sodium ion
NADH-TR	Nicotinamide adenine dinucleotide-tetrazolium.
NaOH	Sodium hydroxide
NCV	Nerve conduction velocity
Neo	Neomycin
NMJ	Neuromuscular junction
NTC	Non template control
OCT	Optimal cutting temperature
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Pgk1	Phosphoglycerate kinase 1
Pgm5	Phosphoglucomutase 5
PNS	Peripheral nervous system

Psmb2	Proteasome subunit beta type 2
PVDF	Polyvinylidene difluoride
Rcan1	Regulator of calcineurin 1
RIN	RNA integrity number
RIPA	Radioimmunoprecipitation assay
rpm	Revolution per minute
rRNA	Ribosomal ribonucleic acid
RT	Room temperature
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SDH	Succinate dehydrogenase
SDS	Sodium docecyl sulphate
SDS-PAGE	Sodium docecyl sulphate-polyacrylamide gel electrophoresis
SEM	Standard error of mean
Sod1	Superoxide dismutase 1
Syjn	*S*ynaptojanin1
TEA	Tris-acetate-EDTA
TEM	Transmission electron microscope
TEMED	Tetramethylethylenediamine
Tm	Temperature
Tmod1	Tropomodulin 1
Tmod4	Tropomodulin 4
UPL	Universal probe library
UV	Ultra-violet
WT	Wild type
Znf295	Zinc finger protein 295

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background

Down syndrome (DS) is a genetic disorder that was first reported by John Langdon Down in 1866 (Down, 1995). The genetic basis of the syndrome remained unknown until 1959 when Lejeune and his colleagues discovered that, the syndrome is due to presence of extra copy of human chromosome 21 (Hsa21), a condition termed as trisomy 21 (Antonarakis *et al.,* 2004). The most common cause of DS is due to non-disjunction; failure of the sister chromatids to separate during cell division (Sherman *et al.,* 2005). What causes non-disjunction that lead to DS is not fully understood but some factors such as genetics, maternal age and altered recombination might play a significant role (Oliver *et al.,* 2008; Sherman *et al.,* 2005). Recent study has also shown association between the non-disjunction and maternal low socio-economic status (Hunter *et al.,* 2013). The prevalence of this disorder is 1 out of every 800 to 1000 live births across different ethnic groups (Egan *et al.,* 2004; Roizen & Patterson, 2003)**.** In some developing countries like Malaysia and Nigeria, the incidence of DS is 1 in every 950 and 865 live births respectively (Adeyokunnu, 1982; Hoe *et al.,* 1989).

DS is characterised with a complex set of pathologies and several clinical phenotypes. There are over 70 phenotypes reported in DS individuals and these include intellectual disability, characteristic sets of facial features, cardiac defect, hypotonia, dementia, anomalies of immune, endocrine and digestive systems (Antonarakis *et al.,* 2004; Lana-Elola *et al.,* 2011; Patterson & Costa, 2005; Wiseman *et al.,* 2009). These phenotypes are due to altered gene dosage in Hsa21 (Korenberg *et al.,* 1994; Nikolaienko *et al.,* 2005). However, not all these phenotypes are seen in every DS individual, although some phenotypes such as intellectual disability and hypotonia are seen almost in all DS individuals (consistent phenotypes) whereas phenotypes such as dementia and systems anomalies are seen only in certain population of DS individuals and are called inconsistent phenotypes (Lana-Elola *et al.,* 2011; Roizen & Patterson, 2003; Wiseman *et al.,* 2009;). Although, the genetics basis of the disorder is the same in individuals with trisomy 21, but the degree of penetrance of these phenotypes varies among different DS individuals. This phenotypic variations are perhaps due to a combined effect of genetic and environmental factors (Reeves *et al.,* 2001; Wiseman *et al.,* 2009) or due to allelic combination (Antonarakis *et al.,* 2004). Moreover, variation occurs in the severity of a particular phenotypes among DS individuals as seen in learning and memory impairment (Lott & Dierssen, 2010) and cognitive impairment (Pennington *et al.,* 2003).

Hypotonia (decreased muscle tone) is seen in most DS individuals (Peredo & Hannibal, 2009). The muscle tone is the normal degree of physical strength and tension maintained in a skeletal muscle by an involuntary spinal reflexes, through which normal posture, coordination and movement are possible (Masi & Hannon, 2008). The skeletal muscle are maintained in a state of partial contraction by the normal muscle tone through an involuntary control and the muscle contracts more forcefully if voluntary impulses are

received. Hypotonia is also seen in many neurological disorders affecting the components of nervous system such as the brain, spinal cord, peripheral nerves or pathways along the motor innervation such as neuromuscular junction (NMJ) and skeletal muscle cells (Bodensteiner, 2008; Leyenaar *et al.,* 2005). The major symptoms of hypotonia includes, difficulties in motor movement, motor planning, postural control, motor coordination, balance and trunk instability (Leyenaar *et al.,* 2005; Peredo & Hannibal, 2009).

Compared with other clinical phenotypes seen in DS individuals, there is not enough literature on hypotonia in terms of its pathophysiological and molecular basis over years. Most of the animal model studies focuse on investigating the neuroanatomical defects and the candidate genes that are responsible for such defects. The area of motor dysfunction received little interest and is considered as neglected area in DS related research. Until recently, a number of candidate genes were implicated in motor dysfunction using *Drosophila* homologs of Hsa21 (Chang & Min, 2009) and in DS individuals (Dey *et al.,* 2013). Similarly, animal based studies have implicated the involvement of some trisomy 21 genes in motor dysfunction using mouse model for DS (Altafaj *et al.,* 2001; Cowley *et al.,* 2012).

Evidence based studies have indicated the involvement of the central nervous system (CNS) disorder (Anderson *et al.,* 2013; Guidi *et al.,* 2008; Guidi *et al.,* 2011; Pinter *et al.,* 2001) and the defective NMJ (Avraham *et al.,* 1988; Chang & Min, 2009) as likely factors in manifestation of hypotonia in DS individuals. However, abnormal organisation of the myofibre components and peripheral nerve defects cannot be ruled out. It will be interesting to investigate the effects of trisomy 21 on the peripheral nerves and skeletal muscle both at cellular and molecular level. Due to ethical limitation in using human as research subject, mouse models for DS are considered as an alternative to investigate this aspect of fundamental research.

There are different mouse models for DS generated over the years (Davisson *et al.,* 1990; Sago *et al.,* 1998) and have shown to exhibit different phenotypes associated with DS individuals (Baxter *et al.,* 2000; Ishihara *et al.,* 2010; Olson *et al.,* 2004; Richtsmeier *et al.,* 2000; Williams *et al.,* 2008). The Hsa21 shared a conserved synteny with orthologous regions of 3 mouse chromosomes, *Mmu10, Mmu16* and *Mmu17* (Galdzicki *et al.,* 2001). These mouse chromosomes contain essentially all the known genes on Hsa21. The *Mmu16* contain the largest number of genes synteny to Hsa21, amounting to about 23.3Mb by length, while the *Mmu17* and *Mmu10* have 1.1Mb and 2.2Mb respectively (Antonarakis *et al.,* 2004). The Ts1Cje is one of the DS mouse models that carries a shorter region of *Mmu16* with approximately 85 genes synteny to genes located on Hsa21 (Antonarakis *et al.,* 2004; Sago *et al.,* 1998). Studies indicated that the Ts1Cje exhibit some features associated with DS individuals (Baxter *et al.,* 2000; Olson *et al.,* 2004; Olson *et al.,* 2007; Sago *et al.,* 1998). The characterisation of phenotypes in this model is still in the process and hypotonia is one of such feature that has not been fully characterised.

To the best of our knowledge and understanding, there has not been any documented research work related to hypotonia on peripheral nervous system (PNS) and skeletal muscle in Ts1Cje mouse model for DS. The PNS and skeletal muscle are actively involved in motor movement and any defects in either can contribute to impaired motor activities. Therefore, since the search for possible causes of the muscle weakness in DS is still on going, investigating the potential role of PNS and skeletal muscle in contributing to hypotonia in DS will be an interesting research. The outcome of this work will further contribute to our current knowledge and understanding of what might have been responsible for muscle weakness in DS. Similarly, in order to formulate a more effective therapy clinically for DS individuals in the future, a better understanding on the development of the hypotonia in DS individuals must be established.

1.2 Problem statement

Motor function have become an integral part of the human life and it is necessary for our daily activities. Delay in motor development due to hypotonia has significant effect in a DS individual's early life style. Inability to properly sit, crawl, walk, run and jump within the normal time frame of a child's physical development due to poor muscle strength will limit the child's interaction with the environment. This in turn will have some consequences on the child's education and general social life. It is clearly evident that muscle weakness in DS is more prominent in children and tends to persist through adult life. With the help of medical and social interventions, the degree of the muscle weakness reduces in adult individuals. However, studies indicated that DS adult individuals also have reduction in physical functions and efficiency, and this have resulted in difficulty to perform certain tasks of life. The overall effect of the muscle weakness in DS individuals leads to limitation in labour productivity and economic self-dependency, which eventually affect the socio-economic status of such individual and the community at large. Therefore, understanding why individuals with DS have low muscle strength is an important area for research that has not yet been explained. An insight of the histological and molecular pathways that lead to hypotonia will have enormous implications for DS individuals' social and medical care needs. A suitable mouse model for DS in studying manifestation of hypotonia will serve as an ideal research platform that leads to a better understanding of the development of this pathological feature.

1.3 Hypothesis

In this study, it is hypothesised that, Ts1Cje mice exhibit hypotonia partly due to histological and molecular disruption of the skeletal muscle and peripheral nervous system (PNS)

1.4 General objective

The general objective is to identify the histological and molecular pathways that contribute to hypotonia in Ts1Cje mice's skeletal muscle and PNS

1.4.1 Specific objectives

This research work have the following specific objectives;

i. to assess the limb muscle strength of Ts1Cje and wild type mice *in vivo*
ii. to investigate the expression profile of some selected trisomic and non-trisomic genes in Ts1Cje and wild type skeletal muscle
iii. to investigate the effect of trisomy 21 on the expression profiles of myogenic regulatory factors (MRFs) on Ts1Cje and wild type skeletal muscle
iv. to evaluate the effects of trisomy 21 on the morphological features and metabolic activities of the skeletal muscle in Ts1Cje and wild type mice
v. to investigate the histomorphology and functional analysis of sciatic nerve in contributing to muscle weakness in Ts1Cje mice

1.5 Significance of the study

This research work is anticipated to have some degree of benefits in the area of DS muscle weakness. The significance of the study include the following:

i. to establish a suitable mouse model to study hypotonia in DS
ii. to reveal the role of some candidate genes and proteins in skeletal muscle that are involved in causing hypotonia in DS
iii. to investigate any pathological defects in skeletal muscle in DS
iv. to find the association of PNS disorder with motor dysfunction in DS
v. finally, to provide an insight for further research work by the clinicians and other allied health professionals that will eventually lead to development of good approach for management of hypotonia in DS individuals

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