STUDIES ON DIABETIC PERIPHERAL NEUROPATHY IN
THE DB/DB, TYPE 2 DIABETES MOUSE

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MBBS

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SUMMARY

In diabetes, many organs and systems develop serious complications, among which diabetic peripheral neuropathy (DPN) is one of the most common. The pathogenesis is still uncertain, and the appropriate choice of experimental models is fundamental in studying this complication. The BKS.Cg-m+/-Leprdb/J (BKS-db/db) type 2 diabetes mouse model has been used commonly since the 1970s. However, the time progression of sequential changes in the peripheral nerves of the db/db model has not been well-defined. We studied the sequential sensorimotor changes in db/db mice from 6 weeks to 26 weeks of age. Nerve conduction velocity (CV), behavioral tail flick and hind paw withdrawal tests were performed. We found that sensory CV delay was detectable at 10 weeks of age, compared to motor CV delay, which was detectable only at 14-16 weeks and varied considerably compared to the sensory CV. We also observed that the peripheral nerve CV increased steadily in non-diabetic controls with age (up to 26 weeks) but in db/db mice, there was no further absolute increase in CV after 6 weeks. There was significant increase in latency in the paw withdrawal response from 6 weeks onwards (P<0.001) but increased latency in tail flick response was detected only from 22 weeks onwards (P<0.05). Therefore, our study indicated that electrophysiological studies may be more consistent and useful as an early diagnostic tool to detect the peripheral neuropathy compared to behavioral tests of reflexes.

The only effective treatment for peripheral neuropathy is good blood glucose control. In this study, we evaluated the therapeutic option of cell therapy for
early DPN in our mouse model. Our earlier study had shown that the sensory system was more suitable as changes were present consistently at 10 weeks. Therefore, we mainly focused on the sensory system in this part of the study and studied the effect of cell therapy from 14 to 22 weeks. There was no significant improvement in the cell treated diabetic mice compared to the saline treated diabetic mice. Direct transplantation of freshly prepared bone marrow cells into diabetic mice was not successful in the treatment of diabetic peripheral neuropathy in db/db mice. Further investigations will be needed, and may include more processing of the bone marrow populations in order to obtain purer stem cell populations.

In conclusion, our study demonstrated that sensory nerve impairment was demonstrable consistently from 10 weeks of age but motor impairment was more variable and demonstrable only at 14-16 weeks of age. In control healthy mice, there was an increase in nerve CV as they grew older but this increase was absent in diabetic mice. This study presents novel information on the development time course on peripheral nerve CV impairment in the db/db mouse model, demonstrating a time difference between sensory and motor CV impairment. This may be important in further studies on the early pathogenesis and early therapeutic intervention in DPN using this mouse model. Further investigations are needed to shed light on cell therapy in diabetic peripheral neuropathy.
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PREVIOUSLY PRESENTED MATERIAL

**NN Thaw Dar, KH Tan, A Chow, Y Guo, G Udolph and E Wilder-Smith.**


Poster: Progression of diabetic peripheral neuropathy in a murine genetic model (db/db mice) of diabetes.

**NN Thaw Dar, KH Tan, A Chow, Y Guo, G Udolph and E Wilder-Smith.**

Presented at 2011 Peripheral Nerve Society Meeting at Bolger Conference Center, Potomac, Maryland, USA

**NN Thaw Dar, KH Tan, A Chow, Y Guo, KO Lee, G Udolph and E Wilder-Smith,**

Characterization of Diabetic Peripheral Neuropathy in a murine genetic model (db/db mice) of diabetes

(Manuscript in preparation)
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AGE</td>
<td>Advanced glycation end-product</td>
</tr>
<tr>
<td>AMDCC</td>
<td>Animal Models of Diabetic Complication Consortium</td>
</tr>
<tr>
<td>ARIs</td>
<td>Aldose reductase inhibitors</td>
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<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
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<td>BMNCs</td>
<td>Bone marrow mononuclear cells</td>
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<td>CMAP</td>
<td>Compound muscle action potential</td>
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<td>CV</td>
<td>Conduction velocity</td>
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<td>DPN</td>
<td>Diabetic peripheral neuropathy</td>
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<td>EPCs</td>
<td>Endothelial progenitor cells</td>
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<tr>
<td>FBG</td>
<td>Fasting blood glucose</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>IDDM</td>
<td>Insulin dependent diabetes mellitus</td>
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<tr>
<td>IGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>MSCs</td>
<td>Mesenchymal stem cells</td>
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<tr>
<td>NCS</td>
<td>Nerve conduction study</td>
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<tr>
<td>NCV</td>
<td>Nerve conduction velocity</td>
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<tr>
<td>NGF</td>
<td>Nerve growth factor</td>
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<tr>
<td>NIDDM</td>
<td>Non-insulin dependent diabetes mellitus</td>
</tr>
<tr>
<td>NOD</td>
<td>Non-obese diabetic</td>
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<tr>
<td>NT-3</td>
<td>Neurotrophin-3</td>
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<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>rhNGF</td>
<td>Recombinant human nerve growth factor</td>
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<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>SD</td>
<td>Standard deviation</td>
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<td>SMCV</td>
<td>Sciatic motor conduction velocity</td>
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<td>SNAP</td>
<td>Sensory nerve action potential</td>
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<tr>
<td>SSCV</td>
<td>Sciatic sensory conduction velocity</td>
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<tr>
<td>STZ</td>
<td>Streptozotocin</td>
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<td>TF</td>
<td>Tail flick</td>
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<tr>
<td>TML</td>
<td>Tail motor latency</td>
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<tr>
<td>TSCV</td>
<td>Tail sensory conduction velocity</td>
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<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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CHAPTER 1

INTRODUCTION
CHAPTER 1: INTRODUCTION

1.1 Diabetes Mellitus

Diabetes mellitus is one of the global epidemics threatening the world population and increasing the cost of health care. The clinical impact of diabetes is high mortality and morbidity, resulting in low quality of patients’ lives and high health care cost. It is estimated that one out of every five health care dollars is spent caring for someone with diagnosed diabetes, while one in ten health care dollars is attributed to diabetes (www.diabetesarchive.net). The prevalence of diabetes for all age-groups worldwide was estimated to be 2.8% in 2000 and 4.4% in 2030 (Wild et al. 2004). The total number of people with diabetes is projected to rise from 171 million in 2000 to 366 million in 2030 (Wild et al. 2004). Not only developed countries but also developing countries have been suffering the burden of diabetes. In developing countries, the majority of people with diabetes are in the age range of 45-64 years and in the developed countries, the majority of people with diabetes are aged ≤65 years (King et al. 1998). These facts highlight that the diabetic epidemic is a growing worldwide concern and requires constant surveillance, and extensive prevention.

Diabetes is characterized by chronic hyperglycemia and a relative or absolute lack of insulin. Depending on the nature of disease, there are two major types of diabetes: type 1 diabetes known as insulin dependent diabetes mellitus (IDDM) and type 2 diabetes known as non-insulin dependent diabetes mellitus (NIDDM). Diabetes can occur temporarily during pregnancy which is called gestational
diabetes. Secondary diabetes may develop as a result of other medical conditions such as chronic pancreatitis, acromegaly, Cushing's syndrome, etc.

**1.1.1 Type 1 Diabetes Mellitus**

Type 1 diabetes (IDDM) is commonly found in childhood and young adulthood (< 40 years) so it is also known as juvenile-onset diabetes, which is approximately 10-15% of all diabetic patients. It is an auto-immune disease in which the immune system attacks the beta cells of pancreas, damaging the source of insulin secretion (Atkinson and Maclaren 1994). Environmental factors such as viral infections, toxins and genetic background are trigger factors of type 1 diabetes. Lack of insulin is the main pathogenesis and therapeutic option is exogenous insulin injection combined with life style control.

**1.1.2 Type 2 Diabetes Mellitus**

Type 2 diabetes (NIDDM) is found in the majority of diabetic patients (85-90%) and is common in adults. It is characterized by insulin resistance which is enhanced by obesity, lack of exercise, poor diet and high blood pressure (Kloppel et al. 1985). Although insulin is still secreted, it cannot function properly to maintain the body metabolic homeostasis resulting in hyperglycemia with hyperinsulinemia. In later stages, beta cells of the pancreas become exhausted and lose their proliferation potential, contributing to a decline in insulin secretion. There is a strong relationship between the degree of obesity and the risk of prevalence of type 2 diabetes (Pi-Sunyer 2002). Life style modification,
anti-hyperglycemic agents and insulin injection are the currently available treatments in type 2 diabetes.

1.2 Diabetic Peripheral Neuropathy

1.2.1 Epidemiology

In diabetes mellitus, hyperglycemia initiates and sustains injury to many organs and systems, resulting in serious complications such as retinopathy, neuropathy, cardiovascular diseases, nephropathy, peripheral vascular diseases and periodontal pathologies (King 2008). Among them, diabetic peripheral neuropathy (DPN) is one of the most debilitating and common complications afflicting about 66% of type 1 and 59% of type 2 diabetic patients (Dyck et al. 1993). Its prevalence rate increases with duration of diabetes and neuropathy symptoms developed in 50% of patients within 25 years of diagnosis (Gundogdu 2006). In the early stage of disease, majority of patients are asymptomatic and only 10% to 18% of patients show abnormality in nerve conduction studies at the time of diabetes diagnosis (Cohen et al. 1998).

1.2.2 Pathogenesis

Metabolic imbalance, vascular defects, and insufficient neurotrophic factors are major roots of DPN pathophysiology and they support each other to trigger the neuronal damage and apoptosis (Gundogdu 2006). Being a metabolic disease,
DPN is initiated by outbalance of glucose control which leads to polyol pathway, advanced glycation end-product (AGE), diacylglycerol, protein kinase C (PKC) and hexosamine pathways resulting in excessive production and insufficient detoxification of reactive oxygen species (ROS) and advanced glycation end-product (AGE) (Brownlee 2001; Gundogdu 2006). ROS and AGE are major toxic substances to kill neurons and schwann cells (Vincent et al. 2004).

Apart from metabolic factors, cardiovascular disease and peripheral vascular pathologies including basement membrane thickening, pericyte degeneration and endothelial cell hyperplasia, increase the risk of diabetic neuropathy. Peripheral vascular changes cause reduction in nerve perfusion, endothelial dysfunction and endoneurial hypoxia (Cameron et al. 2001). Accumulating toxic metabolites (ROS and AGE) resulting from hyperglycemia and hyperlipidemia also enhances endothelial dysfunction and causes the hypoperfusion of peripheral nerves.

In addition, many studies indicate that neurotrophic support plays an important role in repair and regeneration of the damaged neuronal unit. Nerve growth factor (NGF), insulin, insulin-like growth factor 1 (IGF-1), ciliary neurotrophic factor, neurotrophin-3 (NT-3), sonic hedgehog protein, vascular endothelial growth factor (VEGF) and prosaposin-derived peptide are reported to give beneficial support for the regeneration of diabetic peripheral nerves damage (Christianson et al. 2003). Insufficient support of neurotrophic factors is a major problem in neural regeneration of DPN and benefits of exogenous supplement of neurotrophic factors have been investigated in clinical trials of DPN (Apfel 1999).
1.2.3 Types of diabetic peripheral neuropathy

Depending on patterns and types of nerve fiber damage, types of DPN can be classified as follows (Little et al. 2007).

(i). Distal sensorimotor polyneuropathy

It is the most common and widely recognized form of DPN in diabetic patients in which both large and small fibers are affected (Vinik et al. 2000). It is a symmetrical length-dependent neuropathy in which dying-back or dropout feature of the longest nerve fibers – myelinated and unmyelinated is observed (Little et al. 2007). Glove and stock appearance of tingling and numbness sensations, shooting and stabbing pains, hot or cold burning sensations and allodynia are typical symptoms. They are primarily sensory and small fiber dysfunction in the early stage of the disease, and then advancing neuropathy affects large fiber damage resulting in loss of sensation (Little et al. 2007).

(ii). Painful small fiber neuropathy

Small myelinated fibers are mainly affected and patients usually complain of burning or stabbing pain in the lower extremities early in the course of diabetes. Nerve conduction studies may be normal if only small sensory fibers are affected (Little et al. 2007). Painful small fiber neuropathy was observed in impaired glucose tolerance subjects whose prevalence rate is three times higher than age-match population (Singleton et al. 2001). Recent studies reported that painful
small fiber neuropathy presents as an early symptom in the pre-diabetic state of impaired glucose tolerance (Little et al. 2007).

(iii). Acute painful neuropathy

This form of DPN has acute onset and remits over 10-12 months. The symptoms are severe especially at night but the prognosis is good as this can recover. It can be associated with profound weight loss and depression that has been known as diabetic neuropathic cachexia (van Heel et al. 1998).

(iv). Diabetic lumbosacral radiculoplexus neuropathy

It is also known as “diabetic amyotrophy”. The initial symptom is painful sensation in thighs and hip, followed by weakness of the proximal muscles of lower limbs (Vinik et al. 2000). One of the diagnosis tools used to evaluate diabetic lumbosacral radiculoplexus neuropathy is electrophysiological examination and it usually shows motor deficits in the proximal muscle groups (Sander and Chokroverty 1996). Infiltration of inflammatory cells, demyelination and immunoglobulin deposit are detected in the vasa nervorum (Milicevic et al. 1997).
(v). Mononeuropathy

Mononeuropathy is less common than distal sensorimotor neuropathy. Carpal tunnel syndrome, 6th, 3rd and 4th cranial nerve palsies are frequently found in diabetic patients (Little et al. 2007).

(vi). Diabetic autonomic neuropathy

The last form of DPN is diabetic autonomic neuropathy which affects multi-organs and internal systems, including cardiovascular, gastrointestinal, urogenital, sudomotor, respiratory and papillary function which can result in significant morbidity and mortality (Vinik et al. 2003).
1.3 Experimental Mouse Models Used in Diabetic Peripheral Neuropathy

Researchers have extensively investigated DPN for many decades to better understand the basic pathogenesis and therapeutic target. Evidence derived from studies of various animal models of diabetes suggests that DPN is the outcome of complicated sequential interacting and dynamic pathogenetic mechanisms (Brownlee 2001) which may overlap and support each other to go beyond the normal homeostasis mechanism. Gaining extensive knowledge of DPN in diabetic experimental models would serve a first useful platform to better understand the pathogenesis of DPN in humans and shed some light on investigating critical steps in developing clinically useful therapy.

However, there is no well-established DPN experimental model and there are many controversial issues left regarding the wide variation in diabetes induction methods (chemical toxic compound injection or genetic manipulations) and phenotypes of experimental models (molecular and functional features of DPN). These problems still remain as limitations in most DPN studies. Therefore, the choice of an appropriate experimental model is one of the most fundamental keys to explore novel pathological analysis and therapeutic testing in DPN (Leiter 2009). In the field of murine diabetes research, various experimental mice models are available for research, inadvertently generating wide variation in data interpretation. The experimental models range from chemical substance (streptozotocin (STZ)) injected model to genetically manipulated model (BKS-db/db mice, BL6-db/db, ob/ob mice, akita mice, etc.)(Sullivan et al. 2008).
1.3.1 Type 1 diabetic mouse model

Streptozotocin (STZ) induced, alloxan induced, non-obese diabetic (NOD), insulin 1 mutated (Ins.D^1) and insulin 2 mutated (C57BL/6-Ins2^Akita/J) mice are widely used in DPN study of type 1 diabetic research.

Both alloxan (via redox cycle) and streptozotocin (via DNA damage of B cells) cause beta cells necrosis of pancreas by excessive production of reactive oxygen species (ROS), initiators of oxidative stress, resulting in hyperglycemia with low insulin secretion in mice (Szkudelski 2001). Although very few DPN studies use alloxan induced mice, STZ induced mice are widely used (Kyoraku et al. 2009; Serafin et al. 2010; Toth et al. 2010). However, the streptozotocin model sometimes engenders problem associated with maintenance of hyperglycemia for long term. They can recover spontaneously from diabetes by proliferation of beta cells in the pancreas. Regarding DPN features, decrease in thermal sensitivity at 6 weeks after high dose induction (Drel et al. 2007) and delay in motor and sensory nerve conduction velocity at 6-7 weeks after low dose induction (Obrosova et al. 2004; Kellogg and Pop-Busui 2005) are reported. However, thermal latency and nerve conduction velocity (NCV) returned to normal at 24 weeks after low dose induction (Sullivan et al. 2008). This highlights the issue that the STZ model cannot maintain the features of DPN for long term studies.

The genetically modified diabetic mouse model of non-obese diabetic (NOD) mouse was firstly introduced by Makino’s group in 1980. Spontaneous diabetes
was observed due to lymphocyte infiltration into the islets of Langerhan, leading to a decrease in number and size of islets (Makino et al. 1980). In addition, thermal hyperalgesia was reported around 32 weeks of age (Gabra and Sirois 2005) but electrophysiological assessment is not fully explored yet.

Insulin gene mutated diabetic mice, Ins.D1 and Ins2Akita mice are another type 1 diabetic mouse model but it is not widely used in the study of DPN. Moreover, nerve conduction studies and thermal response assessments in this model are not well-defined.

1.3.2 Type 2 diabetic mouse model

Leptin-deficient (ob/ob) model and leptin receptor mutated (BKS-db/db) are commonly used as type 2 diabetic models. The ob/ob model was first introduced at the Jackson Laboratory in 1949 (Ingalls et al. 1950). Leptin-deficient ob/ob mice show significant obesity but relatively mild hyperglycemia (Drel et al. 2006). Motor and sensory conduction deficits, delayed hind paw withdrawal response and reduction in intra-epidermal nerve fiber density were reported at the age of 11 weeks (Drel et al. 2006; Vareniuk et al. 2007).

The BKS.Cg-m+/+Leprdb/J (BKS-db/db) model in which the leptin receptor gene is mutated, is regarded as a robust mouse model for type 2 diabetic neuropathy study because it shows the persistent features of diabetes for long term. BKS db/db mice develop severe DPN and maintain hyperglycemia with standard mouse chow for a long period (Sullivan et al. 2007). Leptin receptor gene
mutation can also be induced in the C57BL6 mouse strain and it is then known as BL6 db/db mice. BL6 db/db mice showed hyperglycemia and neuropathy features only with a high fat diet (Sullivan et al. 2007). However, the mechanism of DPN in type 2 diabetes requires more elaboration since the changes in peripheral nerve functions are widely variable – some groups reported that NCV is slow at 28 weeks (Sullivan et al. 2007) and 33 weeks (Sima and Robertson 1978; Robertson and Sima 1980) but some groups showed that there are no changes in NCV at 20 weeks of age (Whiteley and Tomlinson 1985).

Although pathogenesis, functional and structural analysis of DPN have been extensively explored in the STZ induced type 1 diabetic model, pathophysiological features of DPN in type 2 diabetic model have not yet been well defined. The latter is a major problem because the majority of diabetic patients have type 2 diabetes.

Compared with the db/db model, the ob/ob model has mild hyperglycemia and it mostly represents an obese model. Therefore, db/db may be more relevant and suitable for type 2 DPN study. However, the onset of type 2 DPN features in db/db mice poses a question of “when does the db/db model develop DPN, and which time frame is the best to study DPN?” This is particularly important as early intervention studies are now increasing in number. In our study, we addressed such fundamental questions with nerve conduction study and behavioral tests (thermal sensitivity tests) in the growing mouse.
1.4 Nerve functional assessment of Diabetic Peripheral Neuropathy

1.4.1 Nerve conduction study (NCS)

1.4.1.1 Overview

Nerve conduction study is a test to examine the conduction capability of electrical impulses along motor or sensory or both nerve fibers. It is one of the most important and earliest diagnosis tools in peripheral neuropathy (Morita et al. 2002; Kelly 2004; Higashimori et al. 2005). The main purpose of NCS is to measure the speed and strength of impulses traveling between a defined length of a peripheral nerve and it can confirm the neuropathic defect and further elaborate the type of neuronal impairment (motor or sensory or both) and the pathophysiology (axonal loss or demyelination) (Fricker et al. 2008). Two pairs of electrodes – a pair of stimulating electrodes and a pair of recording electrodes, are required to perform NCS. Electrical stimulus resulting in an action potential is triggered at a specific point, a stimulating point, and the action potential travels along the nerve to the recording site where it is generated as a wave form (Gooch and Weimer 2007). The intensity of stimulation is gradually increased to reach a supramaximal stimulation which depolarizes all axons of the nerve and fully activates them. Compared with human studies, animal studies are more challenging due to the small size of murine bodies and the associated difficulty in handling and managing the animal body as well as the necessary specialized equipment required to perform the study. As much as this kind of study requires technical expertise and manageable skills, the results are still comparable to
those obtained from human (Fricker et al. 2008). It is also one of the reasons why NCS is useful in functional assessment of DPN study in vivo.

1.4.1.2 Interpretation of NCS

The action potential running from the stimulating point is recorded at the recording point and it appears as a waveform on the monitor screen. The time taken to start the action potential is called “latency” of the examined nerve which also partially reflects the conduction speed. In other words, delayed latency can be considered that there would be demyelination defects along the nerve (Gooch and Weimer 2007) because myelin sheath of the nerve serves as an insulator to prevent from the loss of electrical impulse and increase the speed of transmission. However, in the motor NCS, the latency is not as accurate as the conduction velocity because it includes the transmission period across the neuromuscular junction. In motor conduction velocity calculation, the neuromuscular junction transmission period is cancelled out.

Another important component of NCS is the compound muscle action potential (CMAP), which represents the amplitude of the action potential wave. CMAP refers the strength of the action potential. In normal healthy condition, all axons of the nerve are activated and cause depolarization of the innervated muscle fibers once the stimulus is given. The low amplitude of CMAP, a sign of axon loss, indicates the conduction function weakness which is directly related to the interruption of impulses to motor nerves resulting in incomplete depolarization of muscle fibers (Levin 2006). CMAP measured in millivolts, has higher
magnitude than sensory nerve action potential (SNAP) measured in microvolts (Gooch and Weimer 2007). Total duration of the action potential wave is also important to predict the nerve function. In chronic motor axon defects, the activation of axons occurs at different time leading to a longer duration with multiple waves (Gooch and Weimer 2007).

In addition, temperature also highly influences the data of NCS and low temperature makes nerve conduction velocity slow (Levin 2006) because ion channel function, acetylcholinesterase activity, and muscle contractility are temperature related functions (Rutkove 2001). Therefore, in our study temperature was kept constant and monitored frequently during NCS.

1.4.1.3 Motor nerve conduction study

The features of axon loss, demyelination, and defects in neuromuscular junction transmission or severe muscle fiber loss can be detected in motor nerve conduction study (Levin 2006). Electrical stimulations are provided at two sites – one proximal point and one distal point, along the nerve trunk and the action potentials are recorded at only one site over the innerved muscle. Motor conduction velocity is calculated by dividing the “distance” between the proximal stimulating point and distal stimulation point by the “latency” difference between those two points (Kelly 2004; Higashimori et al. 2005). The appearance of motor action potential is biphasic and the latency, CMAP, area and configuration are analyzed to reflect the pathophysiology of DPN.
1.4.1.4 Sensory nerve conduction study

Sensory nerve conduction velocity study is one of the fundamental electrodiagnostic tests to determine the extent of functional impairment in DPN (Bertorini 2006). In sensory nerve conduction study, stimulating electrodes are placed at the distal part and recording electrodes are placed at the proximal part. As the SNAP is formed by integrating of the action potentials of the large myelinated axons of the nerve, while the CMAP is formed by the combination of the individual action potentials of innervated muscle fibers, the magnitude of SNAP is smaller than that of CMAP (Bertorini 2006). Sensory nerve conduction velocity is calculated by dividing the distance between the stimulating and recording points by the latency of SNAP.

1.4.2 Behavioral study

1.4.2.1 Tail flick test

This test has been used as a test of pain sensation in animals to study the effectiveness of analgesic agents since 1941. It is the time taken to flick the tail after a given heat stimulus. It should be noted that the results of the tail flick test can be affected by variation in tail skin temperature and needs to be monitored throughout the test (Berge Og Fau - Garcia-Cabrera et al.).
1.4.2.2 Hind paw withdrawal test

This is a test to determine the thermal sensitivity of diabetic neuropathy. The time taken to withdraw the paw from the source of heat stimulus given by intense radiant heat is recorded. This test provides the quantitative analysis of nociceptive response of unrestrained mice (Hargreaves et al. 1988).

1.5 Therapeutic approaches of Diabetic Peripheral Neuropathy

1.5.1 Glycemic control

Based on the various pathogenetic pathways of DPN, numerous therapeutic approaches can be derived. First of all, glycemic control has been accepted as the best method to prevent and control DPN after decades of experimental trials. The intensive glycemic control reduces the incidence of neuropathy and delays the progression of diabetic complications (Shamoon et al. 1993). However, intensive control is less likely to reverse or regenerate established neuronal injury.

1.5.2 Aldose reductase inhibitors

In addition, many therapeutic studies have been performed by targeting downstream metabolic consequences of hyperglycemia, thereby preventing production of reactive oxygen species, which are believed to contribute to diabetic neuropathy. Inhibition of the increased flux through the polyol pathway
by aldose reductase inhibitors (ARIs) is an interesting strategy for DPN therapy. Trials of ARIs such as Epalrestat, Alrestatin, Tolrestat, Zenarestat, Zopolrestat, NZ-314, Sorbinal, Fidarestat, and AS-3201 (Ranirestat) have been performed over many decades. Although they showed largely negative results over 20 years ago, more recent trials of ARIs (eg, Fidarestat and Ranirestat) that appear to have the greatest efficacy and safest adverse effect profiles, demonstrated improvements in subjective symptoms and electrophysiologic measures, with no improvement in the placebo group (Hotta et al. 2001; Schemmel et al. 2010). Phase III trials of those compounds are in progress.

1.5.3 Antioxidant

Therapeutic strategies that halt oxidative stress, reduce cell injury and restore functional impairments in diabetic complications (Vincent and Feldman 2004). Therefore, antioxidant therapy plays an important role in DPN treatment. The most widely studied antioxidant agent in DPN is alpha-lipoic acid which is approved for the prevention of diabetic neuropathy in Europe (Ametov et al. 2003). Several randomized, placebo-controlled trials have shown that a reduction in neuropathic symptoms such as pain and paraesthesias occurred with short-term use of the intravenous form (Ziegler et al. 1999). Long-term antioxidant therapy trials are underway to evaluate the effects on the progression of DPN.
1.5.4 Neurotrophic support

Deficiency of neurotrophic factors is one of the major causes of chronic ischemic neuropathy and impaired nerve regeneration in DPN. The efficacy of exogenous neurotrophic support (e.g., recombinant human NGF, brain-derived neurotrophic factor) against indices of neuropathy in animal models of diabetes has been reported over decades. However, a randomized, double-blind, placebo-controlled phase 3 trial was conducted from July 1997 through May 1999 to investigate the efficacy and safety of a 12-month regimen of recombinant human nerve growth factor (rhNGF) subcutaneous injection in patients with diabetic polyneuropathy (Apfel et al. 2000). 83% of treatment group and 90% of placebo group completed the regimen. In phase 2 trial, treatment group data showed safe and high efficacy of rhNGF. However, phase 3 trial failed to demonstrate a beneficial effect of rhNGF in treating diabetic polyneuropathy (Apfel et al. 2000).

1.5.5 General comments

The poor results from the many attempts have led to suggestions that earlier intervention may be necessary. In order to conduct early intervention studies, it would be important to document the early development of DPN in the mouse models. This had been relatively neglected in earlier studies as they were more interested in getting a consistent late model to test the efficacy of the treatments.
1.6 Cell Therapy in Diabetic Peripheral Neuropathy

Stem cells have the special ability to self-renew and can differentiate into certain cell types. Therefore, they are an attractive therapeutic source in regenerative medicine. Local transplantation of adult stem cells such as bone marrow mononuclear cells (BMNCs), endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) are used to treat DPN in type 1 diabetes experimental model (Shibata et al. 2008; Jeong et al. 2009; Kim et al. 2009). However, cell therapy in type 2 diabetic neuropathy, which has different underlying mechanisms, is not clear yet.

1.6.1 Bone marrow mononuclear cells (BMNCs)

BMNCs isolated from bone marrow aspirates by density gradient centrifugation are predominantly used to reverse the ischemic tissue injury because BMNCs involve both endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) which are known to induce neovascularization in ischemic insults and secrete a broad spectrum of angiogenic and neurotrophic factors (Kawamoto et al. 2001; Kinnaird et al. 2004; Kim et al. 2009). It has been documented that local transplantation of BMNCs improved DPN of type 1 diabetic experimental model, STZ induced rats, by augmenting angiogenesis and increasing angiogenic and neurotrophic factors in peripheral nerves (Kim et al. 2009). However, the efficacy and long-term effect of BMNCs in type 2 diabetes models are still unknown.
1.6.2 Endothelial progenitor cells (EPCs)

Intramuscular injection of EPCs along the course of the sciatic nerve into STZ induced type 1 diabetic rats to treat DPN showed that EPCs engrafted in the sciatic nerves and increased nerve conduction velocity and neural blood flow by up-regulation of multiple angiogenic and neurotrophic factors at the mRNA and protein levels (Jeong et al. 2009).

1.6.3 Mesenchymal stem cells (MSCs)

Transplantation of MSCs into thigh muscles of STZ induced diabetic rats showed that VEGF and basic fibroblast growth factor (bFGF) mRNA expression were significantly increased in the muscle tissue and hypoalgesia, delayed NCV, decreased nerve blood flow, and decreased axonal circularity of STZ rats were ameliorated by MSCs transplantation (Shibata et al. 2008).

Although both EPCs and MSCs have attractive therapeutic promises in DPN, they are difficult to do in clinical practice because of the complicated procedures needed to isolate cells from bone marrow and grow in culture. However, the ease of BMNCs isolation, in comparison with EPCs and MSCs, makes BMNCs a more feasible source of cells therapy in treatment of DPN. Therefore, we chose unprocessed BMNCs therapy in this study.
1.7 Missing Link and Our Approach

In our study, we focused on characterizing DPN in BKS.Cg-m+/+Leprdb/J (BKS-db/db) mice which have been used in type 2 diabetic research since the 1970s. However, there still remain unanswered questions, regarding peripheral neuropathy profile in this model. Perhaps the most important facts are the fundamental questions of “when does the db/db model develop, and which time frame is the best to study DPN?”. In the current study, we characterized the peripheral neuropathy in db/db by using electrophysiological measurements and behavioral tests.

Moreover, therapeutic efficacy of direct transplantation of BMNCs from donors to recipients is still not well-established in type 2 diabetic model (db/db mice). Therefore, we investigated the safety and efficacy of BMNCs transplantation to treat DPN in db/db mice in our study.

Our present study aims to;

1. To characterize the early development of peripheral nerve functional changes of DPN in db/db mice by monitoring electrophysiological parameters, tail flick and hind paw withdrawal tests.

2. To evaluate early peripheral nerve functional improvement after injecting BMNCs into the muscles along sciatic nerves.
CHAPTER 2

MATERIALS AND METHODS
CHAPTER 2: MATERIALS AND METHODS

2.1 Animals

Genetically mutant homozygous BKS-db/db mice (BKS.Cg-m+/
+leprdb/J) from the Jackson Labs (Jax Stock No. 000642, Bar Harbor, Maine, USA) were used as type 2 diabetic model and heterozygous BKS-db/+ mice were used as healthy control mice. The mice were housed in a pathogen-free environment with a standard mouse-chow diet, water ad libitum, and a fixed 12 hours light-dark cycle was provided. All animal experiment protocols were approved by the Institutional Animal Care and Use Committee of the A*STAR Biomedical Sciences Institute.

2.2 Study design

2.2.1 Study to characterize DPN in db/db mice

Neurophysiological parameters were measured from 6 weeks to 26 weeks of age. 28 homozygous (db/db) mice and 26 heterozygous (db+/) mice, a total of 54 mice, were used in this study. Body weight measurement, fasting blood glucose test, tail nerve conduction study, tail flick test and hind paw withdrawal test were performed in all 54 mice. Sciatic nerve conduction study (NCS) was performed in 13 control mice and 22 diabetic mice (total 35 mice out of 54 mice).
Sciatic NCS was not performed in the first batch of mice due to initial technical problems.

2.2.2 Study to monitor the progress of DPN after BMNCs therapy

In this study, 20 homozygous (db/db) mice and 12 heterozygous (db/+ ) mice, a total of 32 mice, were used to monitor neurophysiological parameters from 14 weeks to 22 weeks of age. Diabetic (db/db) mice were randomly divided into two groups: cells treated group (DM+Cells, n=10) and saline treated group (DM+Saline, n=10). Healthy control db/+ mice were also divided into cells treated group (Cont+Cells, n=6) and saline treated group (Cont+Saline, n=6). The effect of BMNCs was assessed by comparing between DM+cells group and DM+saline group. Whether cell therapy gives adverse effect in normal healthy mice can be evaluated by comparing between Cont+cells group and Cont+saline group.

2.3. Diabetic phenotype assessment

Body weight and blood glucose level were measured biweekly up to 26 weeks of age to ensure the persistence of the diabetic phenotype. Mice were fasted for 6 hours for blood glucose testing according to the established protocols (Sullivan et al. 2007). A drop of blood (10-30μl) was collected from the tail vein under aseptic condition and analyzed with Optium Xceed Glucometer (Abbott diabetes care, USA).
2.4 Peripheral nerves conduction study (NCS)

Tail nerve conduction study (TNCS) and sciatic nerve conduction studies (SNCS) were performed biweekly from 6 to 26 weeks of age, following the guidelines developed by the Animal Models of Diabetic Complications Consortium (AMDCC) (http://www.amdcc.org). Each mouse was anesthetized with 30/2.5 mg/kg ketamine/xylazine intraperitoneal injection (dosage is 0.1 ml per kg body weight) and body temperature was monitored at leg, tail and nape of the neck with a DermaTemp® Infrared Surface Skin Scanner (Exergen Corporation, Massachusetts, USA) and maintained at 32° to 33°C with an infrared radiant lamp. The needle electrodes (12mm in length, 0.4mm in diameter subdermal needles from Viasys Healthcare, USA) were cleaned with 70% alcohol to maintain pathogen-free status and placed into the muscle at predetermined sites.

2.4.1 Tail nerve conduction study

Tail motor nerve conduction study (NCS) was performed by using 2 pairs of electrodes and a ground electrode as shown in Figure 1A. For motor NCS, 2 stimulating electrodes were placed at the base of the tail and 2 other recording electrodes were placed at the distal part of the tail with 30mm interval distance between active stimulating electrode (S1) and active recording electrode (R1). The ground electrode was placed at the space between S1 and R1. The stimulating intensity was increased until the supramaximal amplitude was reached. The motor onset latency and amplitude were then recorded.
To measure tail sensory NCS, the positions of the stimulating electrodes were placed at the distal part of the tail and the recording electrodes were placed at the base of the tail. The distance between S1 and R1 was 30mm and the ground electrode remained unchanged. The onset latency and peak latency were recorded and tail sensory conduction velocity (TSCV) was calculated by dividing the distance (between S1 and R1) by the onset of latency.

**Figure 1 A.** Electrode positions in tail motor nerve conduction study. The red (S2=anode electrode) and blue (S1=cathode electrode) electrodes are a pair of stimulating electrodes, placed at the base of the tail and the white (R1=active electrode) and yellow (R2= reference electrode) are a pair of recording electrodes, placed at the distal part of the tail with 30mm distance interval between active stimulating electrode (blue, S1) and active recording electrode (white, R1). The green electrode (G =ground electrode) was placed between S1 and R1. The 2 pairs of stimulating electrodes and recording electrodes were shifted when the sensory nerve conduction study was performed.
Figure 1 B. Illustration of an actual tracing obtained in tail motor nerve conduction study.

2.4.2 Sciatic nerve conduction study

Sciatic NCS was performed on both the left and the right legs and the average data of two sides was used as result.

Sciatic motor conduction study (SMCS) was recorded at 2 different stimulating sites (sciatic notch and knee) and a fixed recording site (paw) as shown in Figure 2A. The sciatic motor conduction velocity (SMCV) was calculated by dividing the distance between 2 stimulating sites (Proximal – Distal) by the latency difference between two stimulating sites.

Sciatic sensory conduction study (SSCV) was performed by inserting the stimulating electrodes on the hind paw and the recording electrodes at the knee.
The sciatic sensory conduction velocity (SSCV) was calculated by dividing the distance between S1 and R1 by the onset latency.

**Figure 2 A.** Electrode positions in sciatic motor nerve conduction study. The yellow (S2=anode electrode) and white (S1=cathode electrode) electrodes are a pair of stimulating electrodes, placed at the sciatic notch and the blue (R1=active electrode) and red (R2=reference electrode) are a pair of recording electrodes, placed at the paw. The 2 pairs of stimulating electrodes and recording electrodes were shifted when the sensory nerve conduction study was performed.
Figure 2 B. Illustration of an actual tracing obtained in sciatic motor nerve conduction study.

2.5 Behavioral test

2.5.1 Tail flick test

The mouse was placed in an acrylic holder atop a tail flick analgesia meter (Model 336TG Life Sciences, Woodland Hills, CA) so that the tail was in contact with an adjustable red light emitter. The time from activation of the beam to animal response was recorded electronically. The result from each mouse was the average taken from three trials. We followed the guidelines developed by the Animal Models of Diabetic Complications Consortium (AMDCC) (http://www.amdcc.org).
2.5.2 Hind paw withdrawal test

Hind paw algesia was measured using the same apparatus as in the tail flick test. The mouse was placed in compartments on a warm (32°C) glass plate and allowed to habituate for 10 minutes. The light source was maneuvered under the hind paw and the time of activation of the beam to the time of paw withdrawal was recorded. The light source was set at an initial 25°C and the temperature increased to 70°C over the course of 10 sec. A threshold of 70°C is applied to prevent injury to the mice. The guidelines developed by Animal Models of Diabetic Complications Consortium (AMDCC) were applied (http://www.amdcc.org).

2.6 Bone marrow cells extraction and injection

Age matched healthy siblings of diabetic mice were euthanized with over dose of anesthetic drug (ketamine/xylazine). Under aseptic condition, both left and right femur bones were excised and the surrounding tissues were cleaned up. Femur bone marrow was washed out by injecting saline from one end of the femur bone. The extracted bone marrow was washed with normal saline, filtered with 70-μm sieves to obtain cell suspensions, and centrifuged with 2000 rpm for 5 minutes to get cell pallet. The cells pallet was re-suspended with normal saline and cells were counted. Then 1.2 million cells were injected into the muscle of each leg along sciatic nerve.
2.7 Statistical analysis

The distribution of variables for normality was tested with the Shapiro-Wilk test and Q-Q plots analysis and data was presented as mean value at different age. Statistical comparisons between 2 groups were analyzed by non-parametric test, Mann-Whitney U-test, at each time point. Paired t test was used to compare the data of before and after injection. All analyses were performed by using SPSS software version 19 (SPSS Inc., Chicago, IL, USA). Probability values <0.05 (P<0.05) were considered statistically significant.
CHAPTER 3

Characterization of Peripheral Nerves Damage in Type 2 Diabetic Model (db/db mice)
CHAPTER 3: Characterization of Peripheral Nerves Damage in Type 2 Diabetic Model (db/db mice)

3.1 Characterization of Diabetic Phenotype

The assessment of diabetic features was performed biweekly from 6 to 26 weeks of age by monitoring body weight and fasting blood glucose level. These approaches were necessary to confirm that diabetic pathology was consistently and persistently demonstrated in db/db mice over time.

3.1.1 Body weight

Diabetic mice (db/db mice) were obese from 6 weeks of age compared with age matched healthy control heterozygous mice (db/+). Although diabetic mice gained weight progressively until 26 weeks of age, a rapid increase in weight was observed between 6 weeks and 14 weeks. From 14 weeks onwards, the body weight only slightly further increased with age.

In Figure 3, the serial data of body weight of diabetic mice (db/db) and healthy control mice (db+)/ were plotted against the age of the mice. In the healthy control mice, body weight increased slightly with age from 18.48 (+/-1.27) gm at 6 weeks to 26.67 (+/-1.75) gm at 26 weeks. The weight gain of diabetic mice accelerated between the age of 6 weeks (31.95+/-2.56 gm) and 14 weeks (48.19+/-4.24) and then gradually increased up to the age of 26 weeks (49.25+/-
2.33 gm) where the body weight was almost double the weight of healthy age matched control mice (P<0.001).

**Figure 3.** Mean and standard deviation (SD) of body weight of diabetic mice (db/db) and healthy control mice (db/+) at different ages (P<0.001)
3.1.2 Fasting blood glucose

Serial measurements of fasting blood glucose of mice were used to ensure that a consistent and persistent diabetic state was maintained. After both diabetic and healthy control mice were fasted for 6 hours, blood glucose levels were measured. Fasting blood glucose level was monitored throughout the study every two weeks. In Figure 4, the mean and standard deviation (SD) of fasting blood glucose levels of diabetic and control group were plotted against age. Hyperglycemia was observed in diabetic mice (db/db) and was persistent during the entire observation period from 6 (18.53+-5.05 mmol/l) to 26 weeks of age (26.97+-4.77 mmol/l). Fasting blood glucose levels of the control mice was stable between 6.3 mmol/l and 7.5 mmol/l over the whole study period. Fasting blood glucose levels in the diabetic group was significantly higher compared with the age matched control group. (P<0.001)
Figure 4. Mean and standard deviation (SD) of fasting blood glucose levels of diabetic mice (db/db) and healthy control mice (db/+) at different ages (P<0.001)
3.2 Exclusion of Intra-observer's Bias (Test reproducibility)

As mice are small, placing the electrodes can be technically challenging. To investigate intra-operator variability of nerve conduction studies, one 12 week old control and one 12 week old diabetic mouse were tested 3 times alternatively on the same day. The operator was blinded with regard to mouse identity. The data are shown in Table 1. The changes between each test in both the control and diabetic mouse were very small.
<table>
<thead>
<tr>
<th>Mouse</th>
<th>Age</th>
<th>Repetition</th>
<th>Tail motor latency</th>
<th>Tail sensory velocity</th>
<th>Sciatic motor velocity</th>
<th>Sciatic sensory velocity</th>
<th>Temperature</th>
</tr>
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<tr>
<td>Control</td>
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<td>30.96</td>
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<td>28.65</td>
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<td></td>
<td></td>
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<td>0.076376</td>
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</tr>
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<td></td>
<td></td>
<td>AVG</td>
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<td>30.77</td>
<td>28.73333</td>
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</tr>
<tr>
<td>Diabetic</td>
<td>12wk</td>
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<td>25.2</td>
<td>33.97</td>
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<td>32.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2nd time</td>
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<td>23.29667</td>
<td>32.53333</td>
</tr>
</tbody>
</table>

**Table 1.** Retest nerve conduction parameters in a 12 week old control mouse and a 12 week old diabetic mouse
3.3 Tail nerve conduction study

3.3.1 Tail nerve motor conduction study

In this study, a total of 28 diabetic mice and 26 healthy control mice were used and tail nerve conduction function was monitored every two weeks starting from 6 to 26 weeks of age.

The mean values of tail motor latency (TML) of control and diabetic group at different ages are shown in Figure 5. During the observation period, control TML was from 2.5+/-0.22 ms (6 wk) to 1.83+/-0.19 ms (26 wk) and the diabetic group TML was 2.52+/-0.19 ms (6 wk) to 2.42+/-0.37 ms (26 wk). Overall comparison between the control and diabetic group showed that the motor latency of the diabetic group became significantly prolonged compared to that of the control group starting from 14 weeks old (P<0.05).
<table>
<thead>
<tr>
<th>Age (W)</th>
<th>Control group (N=26)</th>
<th>Diabetic group (N=28)</th>
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<tbody>
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<td></td>
<td></td>
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<tr>
<td>8</td>
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<tr>
<td>26</td>
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</tbody>
</table>

**Figure 5.** Mean values of TML in the control and the diabetic group at different ages. Statistical significant difference was observed from 14 weeks onwards. (*P<0.05), ("P<0.01) and (""P<0.001)
However, when the individual TML results of diabetic mice were analyzed, there was considerable variation. TML of some diabetic mice showed no difference from control mice, while TML of some other diabetic mice were prolonged compared to controls. At the end of the study (26 weeks of age), it was observed that 18 diabetic mice (out of total 28 diabetic mice) developed significant neuropathy by showing significant prolongation in TML compared to aged matched controls (2.67+/−0.25ms in diabetic Vs 1.83+/−0.19ms in control) (P<0.001) and their TML was 46% longer than the control TML value. However, 10 diabetic mice (out of total 28 diabetic mice) did not show significant prolong in TML and had very similar TML result compared with control mice (2+/−0.08ms in diabetic Vs 1.83+/−0.19ms in control) (P=NS), Overall their TML was only 9% prolonged compared to that of controls. The mean values of TML data of the control group, the diabetic with neuropathy group and the diabetic with normal TML group were plotted in Figure 6.

From 14 weeks of age onwards, the diabetic with neuropathy group showed significant prolongation of TML compared to the control group (P<0.05) and the diabetic with normal TML group (P<0.05). Therefore, it can be concluded that not all db/db mice developed neuropathy at the same time and the severity of neuropathy among db/db mice had a wide variation.
Figure 6. Mean values of TML in the control group, the diabetic (db/db) with neuropathy group and the diabetic (db/db) with normal TML group

(*P<0.05, †P<0.001)
3.3.2 Tail nerve sensory conduction study

Mean values of tail sensory conduction velocity (TSCV) in the control group and the diabetic group were compared at different time points (Figure 7). Control TSCV was 25.44±1.8 ms⁻¹ at 6 weeks of age, increasing to 36.4±3.1 ms⁻¹ at 26 weeks. Diabetic TSCV was between 24.77±1.8 ms⁻¹ (6 weeks) and 23.4±3.4 ms⁻¹ (26 weeks). The significant delay in TSCV of diabetic mice was detected at 10 weeks onwards compared with the aged matched control group (P<0.05).

Compared to the result of TML, the sensory conduction velocity impairment was observed at the earlier time point and severe decrease in TSCV was detected in all diabetic mice consistently after 10 weeks onwards (P<0.05). At 10 weeks, TSCV of control mice was 30±1.6 ms⁻¹ and that of diabetic mice was 25±2.4 ms⁻¹, and diabetic mice showed 17% decreased compared to controls. At 26 weeks of age, TSCV of control mice was 36.4±3.1 ms⁻¹ and TSCV of diabetic mice was 23.4±3.4 ms⁻¹, and the diabetic mice showed 36% decrease compared to control mice.
Figure 7. Mean values of tail sensory conduction velocity (TSCV) in the control group and the diabetic group

(*P<0.05, †P<0.001)
3.4 Sciatic nerve conduction study

3.4.1 Sciatic nerve motor conduction study

Sciatic motor conduction velocity (SMCV) was monitored biweekly from 6 weeks to 26 weeks of age by using 22 diabetic mice (db/db) and 13 control healthy mice (db/+).

General observation of mean values of SMCV in control group and diabetic group showed that SMCV of diabetic mice became significantly decreased only from 18 weeks onwards (P<0.05). Control SMCV increased from 27.53±1.87 ms⁻¹ at 6 weeks to 46.31±1.81 ms⁻¹ at 26 weeks and diabetic SMCV was 28.33±3.58 ms⁻¹ at 6 weeks and 38.84±6.33 ms⁻¹ at 26 weeks. Sequential SMCV data of both groups are shown in Figure 8. Before 14 weeks of age, diabetic SMCV was unexpectedly faster than control SMCV. However, the SMCV of control mice rapidly increased over time and became faster than that of diabetic after 12 weeks. In diabetic mice, SMCV gradually increased from 6 weeks to 26 weeks but the rate of increase was not as rapid as in controls.
Figure 8. Mean values of sciatic motor conduction velocity (SMCV) in the control group and the diabetic group. A significant difference between 2 groups was found from 18 weeks onwards (*P<0.05, #P<0.01).
However, the development of sciatic motor nerve impairment did not develop equally in all diabetic mice. After tracing the individual mouse data, it was observed that SMCV of some diabetic mice (10 out of total 22 diabetic mice) was similar to that of controls throughout the study (P=NS) and those 10 mice were grouped as “db/db with normal SMCV” in Figure 9. Their SMCV even increased compared to controls’ SMCV at 6-12 weeks. SMCV of some diabetic mice (12 out of total 22 diabetic mice) showed significant decrease compared with the control group (P<0.05) and the “db/db with normal SMCV” group (P<0.05) after 16 weeks onwards and those 12 diabetic mice were grouped as “db/db with neuropathy”. At 16 weeks, SMCV of “db/db with neuropathy” group showed 28% decrease in SMCV and at 26 weeks, the decrease was 32%.

Therefore, it can be concluded that only 55% (12 out of 22 diabetic mice) of the study population of db/db mice showed significant impairment in SMCV at 26 weeks of age.
Figure 9. Mean values of sciatic motor conduction velocity (SMCV) in the control group, the “db/db with neuropathy” group and the “db/db with normal SMCV” group

(*P<0.05, #P<0.01)
3.4.2 Sciatic nerve sensory conduction study

To study the progression of sciatic sensory nerve function, sciatic sensory nerve conduction velocity (SSCV) of 22 diabetic mice and 13 healthy control mice were performed biweekly.

The comparison between control mice and diabetic mice showed that significant SSCV difference started from 10 weeks onwards (P<0.01). Control SSCV increased from 24.1±1.7 ms\(^{-1}\) at 6 weeks to 32.4±1.8 ms\(^{-1}\) at 26 weeks and diabetic SSCV decreased from 23.2±1.14 ms\(^{-1}\) at 6 weeks to 20.4±1.5 ms\(^{-1}\) at 26 weeks. (Figure 10)

At 10 weeks of age, diabetic mice showed 18% decrease in SSCV compared to controls (22.3±1.6 ms\(^{-1}\) in diabetic and 27.1±1.5 ms\(^{-1}\) in control mice). The diabetic SSCV was decreased to 37% at 26 weeks of age compared to controls. Unlike the sciatic motor conduction study, the sensory conduction impairment was detected earlier at 10 weeks of age and all diabetic mice showed a decrease consistently in SSCV after 10 weeks onwards.
Figure 10. Mean values of sciatic sensory conduction velocity (SSCV) in the control group and the diabetic group. The significant difference between 2 group was observed from 10 weeks onwards (*P<0.01, †P<0.001).
3.5 Trends observed in nerves conduction studies

Tail and sciatic nerve conduction studies show that both motor and sensory conduction velocities of control mice increased throughout the observation period. Diabetic mice showed more stable conduction velocities over time. It is worth while noting that, the significant difference between the diabetic group and control group (as shown in Figures 5 to 10) is due to the upward trend of NCV in the controls and is not due to decreasing NCV in the diabetic group.

3.6 Behavioral changes in diabetic (db/db) mice

To study the functional responses in tail nerve and sciatic nerves, the tail flick test and hind paw withdrawal test were performed.

3.6.1 Tail flick response

The tail flick (TF) action involves a combination of sensory response and motor function. Figure 11 shows the time taken to flick the tail from the onset of a heat stimulus recorded from control healthy mice and diabetic mice plotted at different ages. Control TF response was stable over time which was 3+/−0.6 ms (at 6 weeks) and 3.3+/−0.5 ms (at 26 weeks). Diabetic TF response was significantly slower from 22 weeks onwards compared to the age matched control TF response (P<0.05).
During the early age (8 and 10 weeks), TF response of diabetic mice was faster than that of control mice but it was not statistically significant (P=NS).

Figure 11. Mean values of tail flick (TF) response in the control group and the diabetic group. Significant delayed response was observed in diabetic group from 22 weeks onwards (*P<0.05, #P<0.01).
3.6.2 Hind paw withdrawal response

The time taken for the mouse to withdraw the hind paw in response to a heat stimulus was recorded. Each paw was measured three times and the average was taken for calculations. The mean values at different ages are shown in Figure 12. There was a significant and large difference between the two groups from the beginning of testing at 6 weeks. Diabetic mice showed significant persistent slowing from the start at 6 weeks (P<0.001) and this gradually increased with age (from 3.56 +/- 0.71 sec at 6 weeks to 4.57 +/- 0.5 sec at 26 weeks). In control mice, there was a much faster response time, and the slight increase with age from 6 to 26 weeks was not statistically significant (from 2.18 +/- 0.38 sec at 6 weeks to 2.82 +/- 0.34 sec at 26 weeks).
**Figure 12.** Mean values of hind paw withdrawal time in diabetic mice (db/db) and healthy control mice (db+/+) plotted against their age. Statistical difference between diabetic group and control group was observed over all the observation time (P<0.001).
In summary, the earliest abnormality of nerve function in the diabetic group occurred with sensory conduction (10 weeks of age) and motor conduction delay at 14 weeks. Onset and severity of peripheral nerve conduction delay varied considerably among diabetic mice. Motor function of some diabetic mice seems to be normal compared with controls until 26 weeks of age (P>0.05). Slowing in paw withdrawal response was observed from 6 weeks onwards (P<0.001) but delayed tail flick response was detected only from 22 weeks onwards (P<0.05). It is also noted that although nerve conduction studies abnormalities occurred around 10 – 14 weeks, the paw withdrawal test abnormalities occurred earlier since 6 weeks. This finding could be a reflection of the feature of the diabetic distal sensorimotor polyneuropathy in which dying-back or dropout features of the longest nerve fibers are observed. The prolong paw withdrawal latency might be due to the early distal small nerve fibers degeneration which is progressively followed by demyelination and axonal loss of nerve bundles resulting the decreased nerve conduction velocity in the later stage.
3.7 Discussion

3.7.1 Overview

Although the diabetic db/db mouse model is commonly used for the study of diabetic complications, little is known about the time course and pattern of development of peripheral nerve impairment in this model. We used the db/db mice model in our investigation on type 2 diabetic peripheral neuropathy because type 2 diabetic phenotypes such as obesity and hyperglycemia, are consistently observed in db/db mice model. Our study showed that impairment of peripheral nerve conduction varied considerably in both onset and degree of impairment. Generally, sensory nerve impairment started at 10 weeks of age and motor nerve impairment only started at 14-16 weeks of age. Moreover, delay in sensory system was detected earlier and was more severe than motor delay presenting at a later stage. In control healthy mice, an increase in nerve conduction velocity is observed when they grow older, presumably corresponding to continued development of the nervous system until adulthood. However, the lack of such positive improvement was observed in the growing diabetic mice. This is the first study which maps and highlights the onset and severity of peripheral nerve conduction impairment in the growing db/db mouse model and provides the important understanding for further DPN study in db/db type 2 diabetic mice model.
3.7.2 Peripheral nerve functional assessment

In human subjects, many sensory and motor evaluation tools are available (Dyck et al. 1991; Dyck et al. 1995) and functional assessments are easily conducted by following commands and communicating with investigators (Fricker et al. 2008). However, it is difficult to conduct thorough nervous system examination in animal models, considering the smallness of the animal body size, which imposes additional difficulty in handling equipment and lack of ability of test subjects to perform investigator’s commands. Therefore, neuropathy assessment in animal models is mainly based on electrophysiological parameters, behavioral tests and histological analysis (Fricker et al. 2008).

3.7.2.1 Electrophysiological test

In this study, we investigated the progress of electrophysiological changes in sciatic nerves and tail nerves of db/db mice from 6 weeks to 26 weeks of age. Since the 1970s, BKS.Cg-m+/+Leprdb/J (BKS-db/db) mice have been used as a model for study of type 2 diabetic peripheral neuropathy (Sima and Robertson 1978; Sima and Robertson 1979). In DPN research, sciatic nerve conduction study, which is one of the early diagnostic tools for peripheral neuropathies (Kiziltan et al. 2007; Tuncer et al. 2011), was performed in almost all studies of DPN. In our study, sciatic sensory nerve conduction velocity of db/db mice became significantly delayed from 10 weeks of age, comparing to that of control mice (P<0.05). This finding is consistent with the previous report in which delayed sciatic sensory nerve conduction velocity was observed at 28 weeks of
age (Sullivan et al. 2007). Study on sciatic motor nerve conduction velocity showed a significant decrease in velocity after 16 weeks of age (P<0.05). Our finding is in agreement with previous reports on sciatic motor nerve studies that demonstrated similar decrease in CV at 28 weeks (Sullivan et al. 2007) and 33 weeks (Sima and Robertson 1978; Robertson and Sima 1980). In contrast, Whiteley and Tomlinson reported that no obvious changes was detected in db/db mice at 20 weeks of age and they studied only 8 mice in each group (Whiteley and Tomlinson 1985). Our study reconciled this apparent discrepancy in these two reports by demonstrating the age dependent development and inter-individual variation between mice in the onset of sciatic motor nerve impairment in db/db mice. While Whiteley group used only 8 mice in their study, we studied 22 diabetic mice and only 10 out of 22 mice (45%) showed a significant delay in motor conduction velocity at 20 weeks of age. Therefore, the difference in number of diabetic mice studied could account for such a discrepancy in motor nerve impairment. Our larger and more conclusive study therefore showed that the motor function of db/db mice may be normal during the early age in spite of hyperglycemia.

There are few reports for caudal or tail nerve conduction function in db/db mice model. Sullivan et al showed that the tail motor latency was delayed and tail sensory velocity of db/db mice was decreased at the age of 28 weeks (Sullivan et al. 2007). In our study, decreased tail sensory conduction velocity and prolonged tail motor latency were observed starting from 10 weeks and 14 weeks respectively.
Technically, electrophysiological measurements in mice are not as accurate and precise as in humans because of the small size of animals. Despite that, the electrophysiological approaches can provide very useful information comparable to those obtained from humans (Fricker et al. 2008). Decrease in conduction velocity and delay in latency reflects the myelin loss, hypomyelination or demyelination in the diseased models, and a decrease in amplitude of nerve action potential or prolonged duration of action potential is resultant of axon loss in nerves (Gooch and Weimer 2007).

3.7.2.2 Behavioral tests

Behavioral changes are considerably variable and time dependent (Sullivan et al. 2008). Generally, diabetic mouse models exhibit hyperalgesia and allodynia (Anjaneyulu and Chopra 2003; Rashid et al. 2003; Gabra and Sirois 2005) in the early phase, probably around 4 weeks of age, and hypoalgesia (Sullivan et al. 2007; Sullivan et al. 2008) in later phase of neuropathy. In this study, we monitored the sensory perception and pain of db/db mice by conducting two different thermal sensitivity assessment methods such as tail flick and paw withdrawal tests.

In tail flick examination, faster tail flick response (feature of hyperalgesia) was observed at 8 and 10 weeks compared to the response of control mice, but was not statistically significant. Delayed response (feature of hypoalgesia) was observed in diabetic mice compared to controls after 22 weeks which was statistically significant. However, we did not observe hyperalgesia in the paw.
withdrawal test instead hypoalgesia was detectable at 6 weeks of age. We reasoned that our starting time point to detect hyperalgesia might be too late to determine the onset of hyperalgesia with paw withdrawal test, and that both hyper- and hypoalgesia may occur in our study population at the same time point due to individual characteristics of mice models. Recent reports showed that metabolic disorders, peripheral dysfunction and pathology of central nervous system contributed to the disturbed somatosensory perception observed in diabetic animals (Kamiya et al. 2006; Paulson et al. 2007; Piriz et al. 2009).

Compared with electrophysiological tests, behavioral tests are relatively more difficult to interpret and less consistent in animal models since they are influenced by many factors such as emotional factors, outside and skin temperature, and environmental factors.

3.7.3 Time frame of diabetic peripheral neuropathy in db/db mice

All diabetic mouse models are well documented for the continual existence of hyperglycemia. Most of the neuropathy complications studies on diabetic mice were performed only after mice developed DPN. However, there is little literature on investigating the onset of DPN in mice models. On account of this, it is of paramount importance to set the time frame and evaluate the etiology of DPN in diabetic mice model, and as a result, prevent or intervene therapeutically in DPN. To address this problem, we observed DPN development in db/db mice over time, employing nerve conduction velocity examination.
In previous studies, neuropathy assessment in db/db mice was performed only after 25 weeks of age (Moore et al. 1980; Sullivan et al. 2007) and followed up for only a few weeks. Db/db mice showed severe delay in nerve conduction velocity, characteristic of DPN, and thermal hypoalgesia altogether at 25 weeks of age (Sullivan et al. 2007). The profile of nerve functional changes in the early ages of db/db mice is not characterized yet although the db/db model is a robust model to study DPN in type 2 diabetes DPN. On the other hand, type 1 diabetic model, STZ-induced rodents, show the neuropathic complication even 6 to 12 weeks after STZ induction (Kellogg and Pop-Busui 2005; Jeong et al. 2009; Kim et al. 2009).

Complete analysis of the existing experimental model plays a crucial role in understanding the underlying pathology and towards developing effective therapies and preventive approaches (Sullivan et al. 2008). Lack of consistency among mouse models depending on the background strain, diet composition, methods of diabetes induction, and duration of diabetes and lack of consensus concerning phenotype methods are the major obstacles to explore DPN study (Sullivan et al. 2008). Therefore, the Animal Models of Diabetic Complication Consortium (AMDCC) was established to provide the guidelines to identify the appropriate animal models and unify standard protocols to screening DPN in animal models. However, detailed DPN phenotype analyses of db/db mice are still missing. Therefore, in the current study, functional and behavioral biomarkers of neuropathy in db/db mouse model were quantitated over time by using nerve conduction studies and thermal sensitivity tests according to AMDCC guidelines.
Our study showed that sensory conduction impairment began to slow around 10 weeks and motor conduction impairment started around 14-16 weeks of age. We therefore concluded that 16 weeks onwards is a good time point to start therapeutic testing and before 10 weeks is an optimal time point to study the pathogenesis and prevention intervention to DPN in the db/db mice model. Sensory nerve impairment was observed earlier and motor nerve function defect was found in the later stage of diabetes. Our reasoning is that the damage is initiated at the peripheral nerve endings rather than nuclei in the dorso-lumbar region. It is one of the characteristics of distal sensorimotor polyneuropathy in which peripheral nerves are destroyed by drying back features (Little et al. 2007). Previous reports also support this by showing sensory nerves having more severe reduction in fiber size distally, whereas the reverse is true for the motor nerves (Sima and Robertson 1978) and those morphometric changes indicate a disto-proximal progression of the neuropathy on the sensory system (Sima and Robertson 1978).

Furthermore, another interesting finding we observed is the trend of the nerve conduction velocity of healthy control mice and diabetic mice. In all four parameters (tail motor latency, tail sensory velocity, sciatic motor velocity and sciatic sensory velocity) of nerve conduction study from 6 weeks to 26 weeks of age, the trend of NCV is gradually upwards from younger age to older age in control mice, presumably corresponding to arrival of adulthood associated with full-blown development of nervous motor systems. In contrast, such gradual NCV improvement with age was abolished in diabetic mice that showed almost linear trend until 26 weeks. Therefore, at 26 weeks, there is a considerable separation
in NCV between control mice and diabetic mice. It is mainly due to the positive improvement of control mice and not because of the downward trend in diabetic mice. Our finding agrees with the sciatic motor velocity data in the report of Sima’s group (Sima and Robertson 1978) which is the only other study reporting monitoring of NCV over time. They used db/db mice and monitored only sciatic motor conduction velocity at 4 weeks intervals starting from 8 weeks up to 33 weeks of age. They showed that sciatic motor conduction velocity (SMCV) of control healthy mice increased from 33 m/s (8 weeks) to 60 m/s (33 weeks) and that of diabetic mice showed no improvement, fixed at about 30 m/s until 33 weeks. The difference between diabetic and control mice was significant (P<0.001) after 14 weeks (Sima and Robertson 1978). Similarly, the current study showed SMCV increased from 27.5 m/s (6 weeks) to 46.3 m/s (26 weeks) in control mice and diabetic SMCV was about 27 to 31 m/s (Figure 10). We therefore assumed that maturation of myelin sheaths surrounding nerves may account for positive gradual improvement in NCV in control healthy mice because NCV reflects the status of myelin sheath around the nerve fibers (Gooch and Weimer 2007).
3.7.4 Severity level of diabetic peripheral neuropathy in db/db mice

Identification of severity of DPN in patients is essential to conduct the medical practice or epidemiologic surveys or randomized controlled clinical trials (Dyck et al. 2011) and the severity of human patients can be assessed by either stage classification approach (Dyck 1988) or continuous measurement approach (Dyck et al. 2011). However, the severity assessment of DPN in rodent models can only be performed using functional, behavioral or structural examinations.

Like human subjects, diabetic mice showed large variations in results of the nerve conduction functional assessment. Even at the same time point, certain portions of the diabetic mice study population showed severely delayed conduction velocity whereas some diabetic mice showed slightly slower NCV than controls and they seem to be normal. In the current study, the severity of DPN was evaluated in detail by tracing individual NCV. Based on the current data, 55% (sciatic nerve) and 64% (tail nerve) of diabetic mice developed severe motor impairment at 26 weeks of age. This finding highlights that the importance of severity level of DPN in rodents is important to design the therapeutic testing in db/db mice. In addition, different level of severity even in the same strain mice indicates that there may be certain individualistic genetic or molecular mechanisms involved, which may resist to early development of peripheral neuropathy in diabetes. This finding calls for improved knowledge on difference in genetic codes among Homo sapiens, i.e., polymorphisms, and how they play an important role in metabolic pathways as in insulin production and maturation of functional insulin.
3.7.5 Limitations in this study

Being a study in the functional assessment of peripheral nerve in db/db mice, structural and histological analyses were not performed in our study. Nevertheless, our study was able to show that onset of sensory impairment in db/db mice is earlier than that of motor nerve impairment. Further investigation on development of sensory impairment in peripheral nerve and myelin sheath formation could shed some light on our understanding of DPN. Others have reported that the assessment of neuropathy in animal models is improved by additional information of structural analysis of nerves and histological analysis of paw skin (intra epidermal nerve fiber density) (Fricker et al. 2008). This may be a useful continuation of the present study.
CHAPTER 4

Effect of Bone Marrow Cell Therapy in Diabetic Peripheral Neuropathy
CHAPTER 4: Effect of Bone Marrow Cell Therapy in Diabetic Peripheral Neuropathy

4.1 Bone Marrow Cells Injection

In this study, 20 homozygous (db/db) mice and 12 heterozygous (db/+), a total of 32 mice, were used to monitor neurophysiological parameters from 14 weeks to 22 weeks of age. Diabetic (db/db) mice were randomly divided into two groups: cell treated group (DM+Cells, n=10) and saline treated group (DM+Saline, n=10). Healthy control db/+ mice were also divided into cell treated group (Cont+Cells, n=6) and saline treated group (Cont+Saline, n=6). They were monitored up to 8 weeks after cell therapy. The effect of BMNCs was assessed by comparing data between DM+cells group and DM+saline group. Whether cell therapy gives an adverse effect in normal healthy mice can be evaluated by comparing between Cont+cells group and Cont+saline group.

4.2 Confirmation of Diabetic Phenotype in db/db Mice

4.2.1 Body weight and blood glucose

Diabetic (db/db) mice were significantly more obese compared with healthy control (db/+) mice (P<0.001) (Figure 13). Diabetic mice gained weight throughout the study but control mice were nearly stable around 21 to 24 grams in body weight. The body weight of saline treated diabetic group (DM+Saline) was lower
than that of cells treated group (DM+Cells) but it was not statistically significant. Both cell-treated group and saline treated group gained weight similarly throughout the study.

**Figure 13.** Mean and standard deviation of body weight in the four experimental groups (DM+Cells, DM+Saline, Cont+Cells and Cont+Saline group) recorded at before treatment, 2 wk, 4 wk, 6 wk and 8 wk after treatment.
Fasting blood glucose (FBG) levels of four groups are plotted in the Figure 14. Fasting blood glucose of diabetic mice increased progressively throughout the entire observation period. FBG of diabetic mice was significantly higher than that of control mice (P<0.001). It was almost 2.5 times higher than FBG of control mice. DM+Saline group showed higher FBG than DM+Cells group but it was not statistically significant. FBG of both DM+Cells group and DM+Saline group increased over time.

Db/db mice kept the features of diabetes such as obesity and fasting blood glucose during the whole study. Bone marrow cells therapy did not have any significant influence upon the obesity and blood sugar level.
**Figure 14.** Mean and standard deviation of fasting blood glucose level of four experimental groups were plotted at before treatment, 2 wk, 4 wk, 6 wk and 8 wk after treatment.
4.3 Tail Nerve Conduction Study

4.3.1 Motor conduction study

Tail motor latency (TML) is plotted in Figure 15. At the age of 14 weeks, there was no significant difference between the control groups and the diabetic groups. TML of all four groups decreased significantly when comparing before treatment latency time with latency time 8 weeks after treatment (P<0.05). This finding is similar to our earlier finding (see previous data in Chapter 3). The shortening of the latency time was not due to the cell therapy because it was observed similarly in all four experimental groups. At 6 and 8 weeks after injection, DM+Cells group showed faster TML than DM+Saline group but it was not statistically significant. Perhaps, the significant improvement may be observed over a longer period of time.
**Figure 15.** Mean and standard deviation of TML in the four experimental groups starting from before treatment to 8 weeks after treatment.
4.3.2 Sensory conduction study

In Figure 16, diabetic mice (both DM+Cells and DM+Saline groups) showed a significant decrease in sensory conduction velocity that was 25% lower than age matched control mice (both Cont+Cells and Cont+Saline group) (P<0.05) before treatments were given. Severe decrease in tail sensory conduction velocity (TSCV) was observed in diabetic mice at 14 weeks of age (before treatment).

TSCV became slightly increased at the end of the study in all experimental groups. However, there was no significant difference between cell treated diabetic mice (DM+Cells group) and saline treated diabetic mice (DM+Saline group).
Figure 16. Mean and standard deviation of tail sensory conduction velocity (TSCV) of the four experimental groups from before treatment to 8 weeks after treatment.
4.4 Sciatic Nerve Conduction Study

4.4.1 Motor conduction study

Before treatments were given, sciatic motor conduction velocities (SMCV) of diabetic mice were normal and there was no significant impairment compared with healthy control mice (Figure 17). SMCV of diabetic mice fluctuated with wide standard deviation. There were no significant changes between “before treatment” and “after treatment” time points.
**Figure 17.** Mean and standard deviation of sciatic motor conduction velocity (SMCV) in the four experimental groups from before treatment to 8 weeks after treatment.
4.4.2 Sensory conduction study

Sciatic sensory conduction velocities (SSCV) of 4 experimental groups were plotted in Figure 18. Severe sensory conduction velocity decrease; 26%; was observed in diabetic mice compared to that of control mice, before treatments were given (P<0.05). Both control groups increased in their SSCV regardless of cell treatment or saline treatment at the end of the study (25.44+-1.91 to 31.31+-2.06 ms\(^{-1}\) in the Cont+Cells group and 25.96+-0.59 to 31.72+-1.38 ms\(^{-1}\) in the Cont+Saline group). However, diabetic groups showed a more stable SSCV over time. There was no significant improvement in cells treated diabetic mice compared with the saline treated diabetic mice.
Figure 18. Mean and standard deviation of sciatic sensory conduction velocity (SSCV) in the four experimental groups from before treatment to 8 weeks after treatment.
4.5 Behavioral Tests

4.5.1 Tail flick test

Thermal sensitivity of tail nerve was assessed in the four experimental groups (Figure 19). Diabetic groups showed a slightly delayed response compared with control groups before treatments were given, but it was not statistically significant. Tail flick latency of control groups was ranging from 2.7 to 3 ms but that of diabetic groups fluctuated widely through out the study. No significant change in result was observed in the cell treated diabetic group.
**Figure 19.** Mean and standard deviation of tail flick test in the four experimental groups from before treatment to 8 weeks after treatment.
4.5.2 Hind paw withdrawal test

Hind paw withdrawal tests recorded in four experimental groups were plotted in Figure 20. Significantly delayed hind paw responses were observed in diabetic mice compared with control mice (P<0.05) before treatments were given. Only at 8 weeks after injection, DM+Cells group became faster than DM+Saline group (4+/-0.75 vs 4.6+/-0.99 ms) but it was not statistically significant. Therefore, no positive improvement of cell therapy was observed in diabetic mice.
Figure 20. Mean and standard deviation of hind paw withdrawal test in the four experimental groups from before treatment to 8 weeks after treatment.
4.6 Discussion

The present study demonstrated that local injection of bone marrow mononuclear cells (BMNCs) along the sciatic nerve did not show any significant improvement in nerve conduction function and thermal sensitivity analysis of db/db mice. 14 weeks old db/db mice were used in this study and nerve conduction function impairment was observed only in the sensory system. The motor function seemed to be normal and did not show any difference compared with healthy control mice. This interesting finding led us to evaluate the progress of peripheral nerves functional impairment in db/db mice which is explained in chapter 3. From our previous data, sensory deficit was significantly more severe at 14 weeks of age whereas motor function was spared at that time.

Thermal sensitivity tests showed that significant prolonged latency was observed in the tail flick test and the hind paw withdrawal test of diabetic mice but bone marrow cell transplantation did not ameliorate that abnormality.

Previous reports have studied the effect of transplantation of BMNCs (Kim et al. 2009), EPCs (Jeong et al. 2009) and MSCs (Shibata et al. 2008) on peripheral neuropathy in type 1 diabetic rodent models, usually in STZ induced diabetic rats. These reports have extracted cells from the donor rodents, performed fluorescent labeling on the cells and injected these cells into the recipient rodents. Their reports showed that local transplantation of BMNCs, EPCs or MSCs improved experimental DPN by augmenting angiogenesis and increasing angiogenic and neurotrophic factors in peripheral nerves (Shibata et al. 2008;
Jeong et al. 2009; Kim et al. 2009). In their studies, STZ-induced diabetic rats, a type 1 diabetic model, were used and significant functional impairment was observed in the model 12 weeks after induction of diabetes. Our study is the first study to test cell therapy in a type 2 diabetic model, db/db mice, in which features of neuropathy are not demonstrable at 14 weeks of age.

Apart from the usage of different animal models for different types of diabetes, the major difference between these previous reports and the current study is the method of BMNCs isolation. In the study of Kim’s group (Kim et al. 2009), they characterized BMNCs by using flow-cytometric analysis and the expression of hematopoietic cell markers was observed in BMNCs as followings: CD45 (99%), CD3 (3%), CD45R (79%) and CD11b (8%). Then they labeled BMNCs and injected them into the mice. It is documented that bone marrow cells are the major source of adult stem cells such as EPCs and MSCs (Kim et al. 2009). In order to avoid the contamination contingent upon culturing extracted cells in bovine serum, labeling cells, extra steps in isolating cells, we took an approach that would be simple and feasible in clinical practice, and investigated if this simpler approach would produce similar results. In our study, we extracted the cells from the femur, washed the cells with normal saline, filtered with 70-μm sieves to obtain cell suspensions, centrifuged to get cell pellet, and injected such freshly prepared cells into the mice. We thereby avoided the in vitro culture system and contamination hazard of bovine serum, which is commonly used in cell culture systems and can give immune rejection problems in cells transplantation. Therefore, we tested the direct transplantation of freshly prepared bone marrow cells therapy in this study. We observed no significant
improvement after direct transplantation of bone marrow in this study. Therefore, we concluded that processing techniques are a necessary requirement for efficacy, and the simple injection of freshly extracted bone marrow cells, while safe and easy to perform, may not be effective.
CHAPTER 5

CONCLUSIONS
CHAPTER 5: CONCLUSIONS

In conclusion, C57BL/Ks db/db mice demonstrate abnormality in peripheral nerve functions at different times of duration of diabetes. The following important facts may help us to better understand the pathogenesis and therapeutic targets of diabetic peripheral neuropathy in future studies.

1. Sensory conduction impairment started around 10 weeks and motor conduction impairment started around 14-16 weeks of age.

2. It can be concluded that 16 weeks onward is a good time point to start therapeutic testing and before 10 weeks is an optimum time point to study the pathogenesis and prevention intervention to DPN in db/db model.

3. Sensory impairment is demonstrable earlier and is more severe than motor impairment in this model.

4. Nerve conduction velocity of healthy control mice becomes faster when the mice are growing older from 6 weeks to 26 weeks. However, that positive improvement is abolished in diabetic mice.

Direct transplantation of freshly prepared bone marrow cells into diabetic mice is not effective in improving diabetic peripheral neuropathy in db/db mice. Further investigations are needed to refine the method of cell delivery to the
specific tissue location with minimal manipulation during purification of
required cell population to obtain safe and effective cell therapy.
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