

Lumbricus rubellus Protein Fraction DLBS1033N Increases Nerve Growth Factor Expression via Tyrosine Kinase Activation

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Abstract: Nerve injury occurred due to mechanical, thermal, chemical, or ischemic factors. Nerve regeneration is needed for recovery. Schwann cell proliferation and differentiation are important factors in the nerve regeneration process. Schwann cells release neurotrophins in the nerve regeneration process. In this study, *Lumbricus rubellus* protein fraction DLBS1033N was used as therapeutic protein candidate for nerve regeneration treatments. DLBS1033N treatments promoted the growth and survival of Schwann cell in free serum and free serum plus minimum O₂ conditions. Real-time PCR and ELISA methods revealed that DLBS1033N induced NGF expressions. The growth and survival of Schwann cells were related to NGF expressions in a specific inhibitor TrkA study. Furthermore, in real-time PCR study, DLBS1033N was able to activate phosphatidylinositol-3-kinase (PI3K) pathway. This study showed that *L. rubellus* protein fraction DLS1033N can promote the growth and survival of Schwann cells by inducing NGF expressions. Cells growth and survival activities are likely achieved via PI3K pathway.

Keywords: *Lumbricus rubellus*, Schwann Cell, Peripheral Nerve Regeneration, Cell Survival, NGF

Introduction

Nervous system can be divided into central nervous and peripheral nervous system. Both systems have different anatomical structure and regeneration abilities. Nerve could be injured due to mechanical, thermal, chemical, or ischemic factors (Zhang *et al.*, 2005). Nerve regeneration is needed when the nerve is degenerated or injured (Bunge, 1993). However, nerve regeneration is more possible in the peripheral nervous system due to the anatomical structure and the necessary biochemical reaction (Chen *et al.*, 2007). Schwann cells are a part of the peripheral nervous system involved in nerve regeneration process. When the peripheral nerve injury occurred, Schwann cells with macrophages were assisted in the removal of axonal and myelin debris (Bunge, 1994). Axon regeneration and remyelination are needed for nerve regeneration process. The Schwann cells will differentiate and proliferate in order to form Bungner bands that will allow axon regeneration and ensue remyelination after injury (Bunge, 1993; 1994; Ide, 1996).

Another important factor in peripheral nerve regeneration process is neurotrophins which are the regulators of neural survival, development, function and plasticity (Bunge, 1993; 1994; Ide, 1996; Huang and Reichardt, 2001). Some neurotrophins such as Nerve Growth Factor (NGF) and Brain Derived Neurotrophic Factor (BDNF) are produced by Schwann cells. Neurotrophins are released around regenerating axon (Ide, 1996). At least four neurotrophins have been well characterized in mammals: NGF, BDNF, neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4). The neurotrophins bind two types of receptor, tyrosine kinase receptors and low-affinity NGF receptor p75 (Hallbook, 1999). NGF binds specifically to TrkA; BDNF and NT-4 bind specifically to TrkB; and NT-3 binds to TrkC (Huang and Reichardt, 2001; Hallbook, 1999; Lewin and Barde, 1996).

Therapeutic proteins have been increased in numbers and uses since the introduction of recombinant insulin therapy. Therapeutic proteins can be obtained from their native sources or from recombinant technology (Leader *et al.*, 2008). Earthworm is one example of the

source of therapeutic protein extracted from their native source. Lumbrokinase is a common therapeutic protein obtained from earthworm that possess thrombolytic effect (Mihara *et al.*, 1989; 1991; Cho *et al.*, 2004; Verma and Pulicherla, 2011). However, reports showed that earthworm extract also possessed other activity, in addition to its thrombolytic effect. Study by Chang *et al.* (2011a; 2009) showed that earthworm extract exhibited good activity on peripheral nerve regeneration.

In this study, earthworm *Lumbricus rubellus* protein fraction DLBS1033N was used as therapeutic protein candidate for peripheral nerve regeneration treatments. The experiments were designed to observe DLBS1033N activity on Schwann cell growth and survival in free serum and free serum-with minimum O₂ conditions. The conditions were prepared for mimicking hypoxia-ischemic state. In this study, the experiments were also designed to investigate DLBS1033N activity in inducing neurotrophins.

Materials and Methods

DLBS1033N Preparations

Protein fraction DLBS1033N was made using *Lumbricus rubellus* protein fraction DLBS1033 provided by Dexa Laboratories of Biomolecular Sciences (DLBS) (Trisina *et al.*, 2011). DLBS1033 (1 mg mL⁻¹) was hydrolyzed with heat (100°C) in a water bath for 30 min. The liquid (\pm 1 mg mL⁻¹) was then sterilized with 0.22 μ m filter before the use for cells treatments.

Materials

TrkA inhibitor, GW441756 was purchased from Sigma (MO, USA). Oxygen absorber Anaerocult® was purchased from Merck (Darmstadt, Germany). Collagen for neurite outgrowth assay was purchased from Sigma (MO, USA).

Cell Culture

Schwann cell line *Rattus norvegicus* RSC96 (ATCC® CRL2765™) and adrenal gland *Rattus norvegicus* cell line PC-12 (ATCC® CRL1721™) were purchased from American Type Culture Collection (Rockville, MD, USA). The RSC96 cells were cultured in DMEM media with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin (Gibco, Grand Island, USA). The PC-12 cells were cultured in RPMI 1640 media with 10% Fetal Bovine Serum and 1% Penicillin/Streptomycin (Gibco, Grand Island, USA). The RSC96 and PC-12 cells were cultured in 37°C, 5% CO₂ conditions.

Cell Proliferation Assay

To do the cell proliferation assay in free serum condition, RSC96 cells were cultured in 96-well plate at 1×10³ cells/well. Meanwhile, to do cell proliferation in

hypoxia-ischemic condition, RSC96 cell were cultured in 6 cm petri dish at 2×10⁵ cells per petri dish. RSC96 cells were treated with 25, 50, 75 or 100 μ g mL⁻¹ DLBS1033N for 24, 48 or 72 h. For hypoxia condition, RSC96 cells were incubated with free serum media for 4 h before cultured in sealed beaker glass containing oxygen absorber Anaerocult®. RSC96 cells were incubated for 24 h with 50 μ g mL⁻¹ DLBS1033N. Cell viability was observed using CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay Reagents (Promega, Fitchburg, WI, USA).

FACS Cell Analysis

RSC96 cells were cultured in 6-well plate at 1×10⁵ cells/well. In the following day, the culture media was exchanged with free serum media and incubated for 4 h prior to any treatment. RSC96 cells were treated with 50 μ g mL⁻¹ DLBS1033N for 24, 36 or 72 h, then harvested (either live or death cells) into pellets. The cell pellets were washed with PBS and fixed with 300 μ L PBS and 700 μ L ethanol. The sample was incubated at 4°C for at least 24 h before running in FACS machine. After incubation, the fixed cells were centrifuged to obtain the cell pellets. Propidium iodide was added to the cell pellets for 1 ml and further incubated for 30 min in dark conditions. The sample was run in FACS machine (BD, San Jose, CA, USA) and analyzed with Cell Quest program (BD, San Jose, CA, USA).

RNA Preparation and Real-Time Assay

RSC96 cell were cultured on 6 well plate at 1×10⁵ cells/well. In the following day, free serum media was administrated for 4 h prior to the treatments. RSC96 cells were treated with 50 μ g mL⁻¹ DLBS1033N for 48 h. Total RNA was isolated using Trizol reagent (Invitrogen, CA, USA). The RNA concentration was quantified using NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA, USA). Reverse transcription of total RNA (1 μ g) was done using GoScript reagent from Promega (Fitchburg, WI, USA). Expression was carried out using gene-specific primers

NGF	F:	5'-
	R:	5'-
FGF	F:	5'-CGT
	R:	5'-
GDNF	F:	5'-GCC
	R:	5'-
BDNF	F:	5'-GGC
	R:	5'-
PI3K	F:	5'-
	R:	5'-TCT
Akt	F:	5'-
	R:	5'-GAT
BAD	F:	5'-
	R:	5'-CAC
BCL-2	F:	5'-
	R:	5'-GCA
BCL-xL	F:	5'-

AGCAGTCAGCCAGAACCCTA-3', R: 5'-GAG
ATGGGCTCAACCAGTCC-3'; MAPK F: 5'-
TCACAGACTGATGCTCTGGGT-3', R: 5'-CTA
CTGAAGGCTAGTGGTGGG-3'; CREB F: 5'-
CTGAGGAGCTTGTACCACCG-3', R: 5'-GGC
TGGGCTTGAACCTGTCAT-3'; C-FOS F: 5'-
GGTTTCAACGCGGACTACGA-3', R: 5'-GCG
CAAAAGTCCTGTGTGTT-3' and C-MYC F: 5'-
AACAACCGCAAATGCTCCAG-3', R: 5'-AAG
CTACGCTTCAGCTCGTT-3'. qPCR process were done
using SsoFast™ EvaGreen® Supermix (Biorad, Hercules,
California, USA). The qPCR was performed using Mini
Opticon MJ Mini™ (Biorad, Hercules, California, USA) at
the optimum conditions for each primer.

NGF ELISA Assay

RSC96 cells were cultured in 6 cm petri dish at 2×10^5 cells/well. In the next day, the free serum media was given to the cells for 4 h prior to treatments. RSC96 cells were treated with $50 \mu\text{g mL}^{-1}$ DLBS1033N for 48 h. Total protein from media was concentrated using Amicon® Ultra-15 (MerckMillipore, Darmstadt, Germany) from 2 mL to 200 μL . NGF in media was investigated using ChemiKine™ NGF ELISA kit (Merck, Darmstadt, Germany) according to the manufacturer's protocol.

Neurite Growth Induction

Neurite growth assay was adapted from Smit *et al.* (2003) and Yang *et al.* (2014) with some modifications. Twelve-well plate were prepared for neurite growth induction. The plate was coated with extracellular matrix reagents containing collagen/PBS, 0.3 mg mL^{-1} , for 4 h. PC-12 cells were plated on 12-well plate for 5×10^4 cells/well. The PC-12 cells were treated with $50 \mu\text{g mL}^{-1}$ DLBS1033N for 72 h.

Statistical Analysis

Data are presented as the mean \pm Standard Error (S.E.). Significant differences between experimental values were determined using student t-test with $p < 0.05$ considered as significance.

Results

DLBS1033N Induces Growth and Survival in Free Serum and Free Serum-Minimum O_2 Conditions

In this study, RSC96 (ATCC® CRL2765™) Schwann cell was used. The cell was prepared in two conditions, which consisted of free serum media for up to 72 h, and free serum media and minimum O_2 condition for 24 h.

For free serum condition, the DLBS1033N was administrated to the cell with concentrations of 25, 50, 75 and $100 \mu\text{g mL}^{-1}$. Cell growth was observed after 24, 48 and 72 h incubation. The result showed that sample

treated with DLBS1033N exhibited more cells populations detected compared to the control (Fig. 1). At 24 h after treatments, the cells populations were almost identical in number between the positive control (DMEM (+) 10% FBS), control (DMEM (-) serum only) and sample treated with DLBS1033N. After 48 h of treatments, the control sample exhibited fewer cells populations compared to the positive control. Cells treated with DLBS1033N were proliferated almost identical to the positive control. The higher cells populations were found on sample treated with $50 \mu\text{g mL}^{-1}$ DLBS1033N compared to the control sample. After 72 h, the positive control sample showed higher cells population compared to the control and DLBS1033N-treated sample. However, cells populations were higher in DLBS1033N samples compared to the control. Other protein used as a negative control was Bovine Serum Albumin (BSA). The experiments were done for 48 h with concentrations of DLBS1033N and BSA 25 and $50 \mu\text{g mL}^{-1}$. The results showed that only DLBS1033N-treated samples have more cells populations compared to the control sample (Fig. 2).

Following FACS analysis result, it was found that S phase in sample treated with $50 \mu\text{g mL}^{-1}$ DLBS1033N on 24, 36 and 48 h were higher than that of the control sample (Fig. 3). After 24 h, the S phase of control sample and treated sample were 6.8% 14.0%, respectively. Moreover, after 36 h, the S phase of control was 7.4% and the treated sample was 10.1%. At 48 h, S phase of control was 7.1% and the treated sample was 8.2%.

The study was continued with growth and survival assay with free serum and minimum O_2 conditions for hypoxia-ischemic mimicking condition. The RSC96 cells were treated with $50 \mu\text{g mL}^{-1}$ DLBS1033N and prepared for the minimum O_2 conditions. The plate was sealed with parafilm and inserted into beaker glass containing Anaerocult® then sealed with aluminum foil and parafilm. RSC96 cells were incubated for 24 h. The study shows that RSC96 cells treated with $50 \mu\text{g mL}^{-1}$ DLBS1033N were proliferated and survived (Fig. 4).

The results showed that control sample (minimum O_2) in hypoxia-ischemic conditions has fewer cells populations compared to the control sample in normal conditions. Meanwhile, cells populations treated with $50 \mu\text{g mL}^{-1}$ DLBS1033N in hypoxia-ischemic condition was survived and proliferated. The cells populations were higher than that of control in normal conditions.

DLBS1033N Induces NGF Expressions

In order to determine the mechanisms of action of the growth and survival activity, gene expression of several neurotrophins and growth factors were investigated using real-time PCR method. The neurotrophins and growth factors studied were NGF, FGF, BDNF and GDNF. The cells were treated with $50 \mu\text{g mL}^{-1}$ DLBS1033N for 48 h. The investigation result is shown in Table 1.

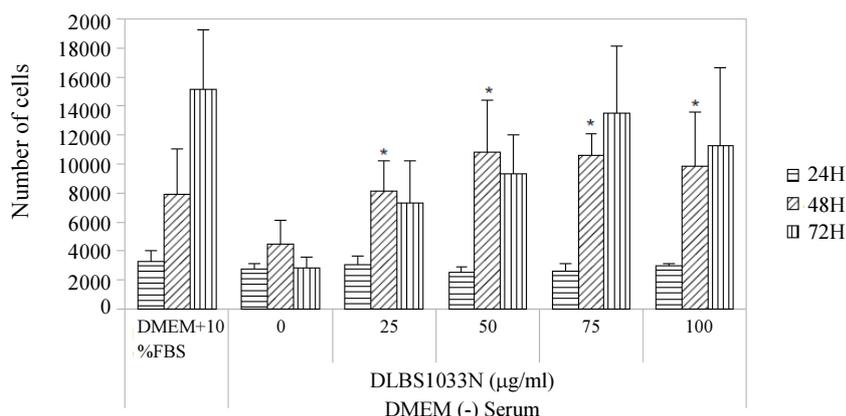


Fig. 1: Cell growth and survival in free serum conditions, RSC96 cells were treated with various concentration of DLBS1033N for 24, 48 and 72 h. Values are expressed as means \pm S.E. of eight independent experiments (* $p < 0.05$, compared with $0 \mu\text{g mL}^{-1}$ DLBS1033N)

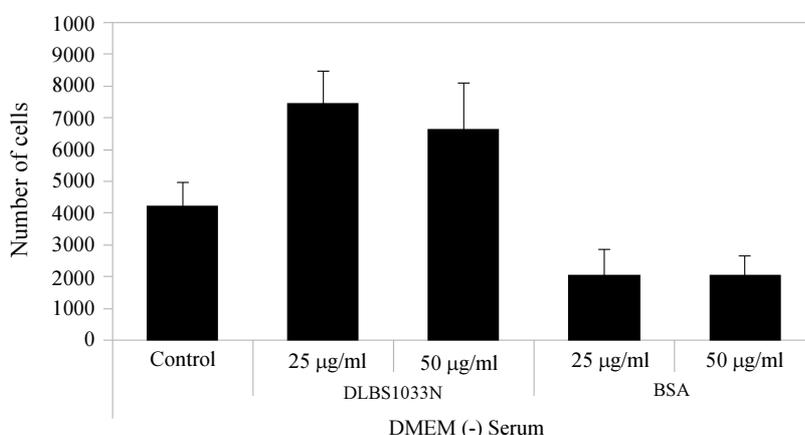


Fig. 2: Other protein (BSA) did not induce growth and survival of RSC96 cells, RSC96 cells were treated with DLBS1033N or BSA 25 and $50 \mu\text{g mL}^{-1}$ in free serum media. Some samples were also treated with GW441756 $20 \mu\text{g mL}^{-1}$. Values are expressed as means \pm standard deviation of eight independent experiments (* $p < 0.05$, compared with control)

Table 1: Fold change of several growth factor gene expressions

Gene	Fold change (48H) Sample		
	Control	DLBS1033N ($50 \mu\text{g mL}^{-1}$)	Std. Deviation
NGF	1	1.56	0.51
FGF	1	0.53	0.08
BDNF	1	0.37	0.02
GDNF	1	0.52	0.16

The results showed that only NGF gene was up-regulated by $50 \mu\text{g mL}^{-1}$ DLBS1033N at 48 h incubation. The other neurotrophins and growth factor tend to be down-regulated. It was found that $50 \mu\text{g mL}^{-1}$ DLBS1033N could up-regulated NGF gene expressions by almost 50% more than the control. To confirm the action of DLBS1033N in inducing NGF, further study which observed NGF expression at protein level was conducted. The NGF protein was secreted by the cells. Therefore, to investigate NGF protein expressions, the

protein in cell media was analyzed with NGF ELISA kit. NGF protein in the cell media from the DLBS1033N-treated sample was detected (Fig. 5). The NGF-ELISA study revealed that NGF protein in cell media was increased in a time-dependent manner. The NGF concentration on day 0 was $35.0 \mu\text{g mL}^{-1}$. On day 1, the NGF protein concentration was increased up to 35.5% (compared to day 0) to $47.4 \mu\text{g mL}^{-1}$. On day 2, the NGF protein was detected and increased up to 45.9% (compared to day 0) to $51.1 \mu\text{g mL}^{-1}$.

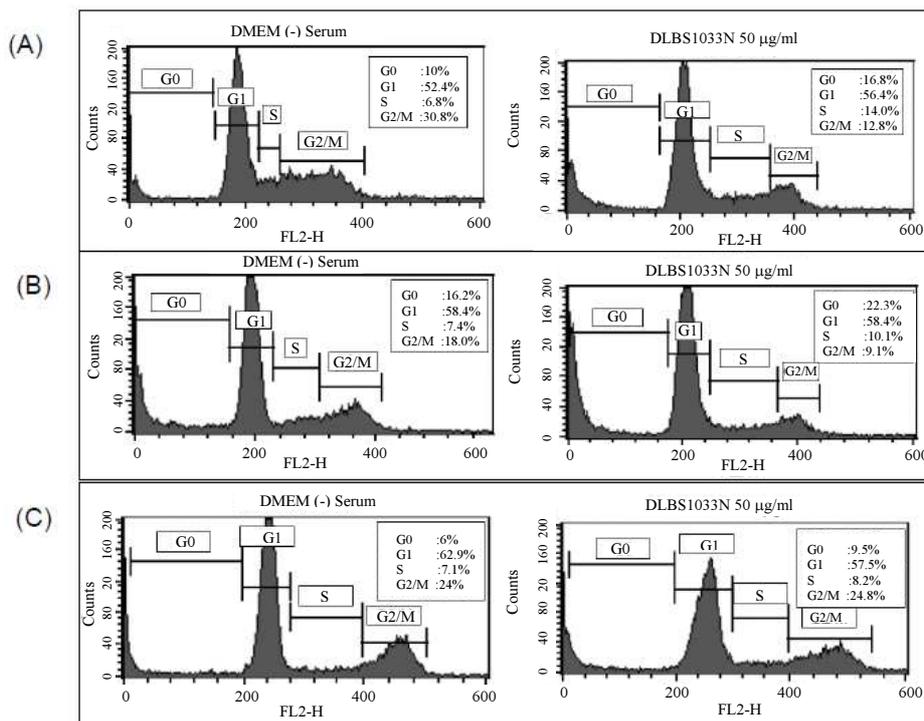


Fig. 3: Cell cycle analysis in RSC96 cells after treated with DLBS1033N, Cell cycle analysis was performed in RSC96 cells after treated with DLBS1033N 50 µg mL⁻¹. (A) Peak distribution of each phases G0, G1, S and G2/M after 24 h treatments. (B) Peak distribution of each phases G0, G1, S and G2/M after 36 hours treatments. (C) Peak distribution of each phases G0, G1, S and G2/M after 72 h treatments

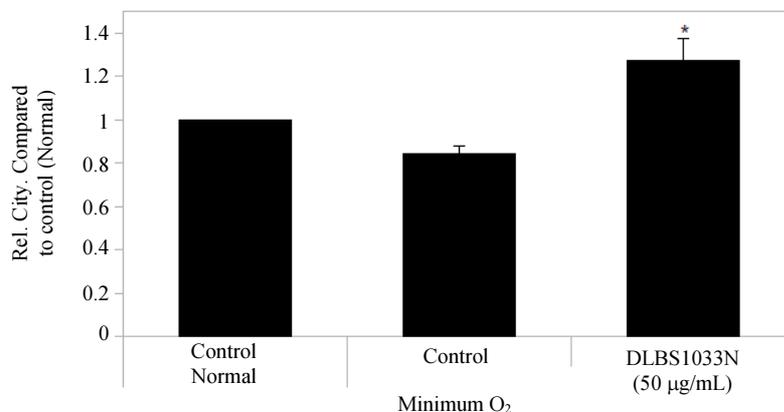


Fig. 4: Cell growth and survival assay in free serum-minimum O₂ conditions, Cell survival at minimum O₂ condition assay was conducted on RSC96 cell treated with 50 µg mL⁻¹ DLBS1033N for 24 h. Values are expressed as means ± S. E. of two independent experiments (*p<0.05, compared with control-normal)

In order to confirm the action of DLBS1033N on growth and survival through NGF, receptor inhibition study was conducted. The RSC96 cell was treated with 50 µg mL⁻¹ DLBS1033N. Inhibitor GW441756 was introduced into some of the DLBS1033N-treated cells with concentrations of 2.5, 5, 10 and 20 µg mL⁻¹. Sample treated with GW441756 and DLBS1033N exhibited less cell population compared to DLBS1033N treatment only (Fig. 6). The result showed that

DLBS1033N treatment (50 µg mL⁻¹) resulted in higher cells populations compared to the control. However, when GW441756 was introduced, the cell growth was inhibited. The inhibition appeared as a dose-dependent manner. The higher GW441756 introduced to the DLBS1033N treated cell, the less cell growth effect was detected. The cell growth was inhibited up to 43.9% by 20 µg mL⁻¹ GW441756 (compared to DLBS1033N treatment only).

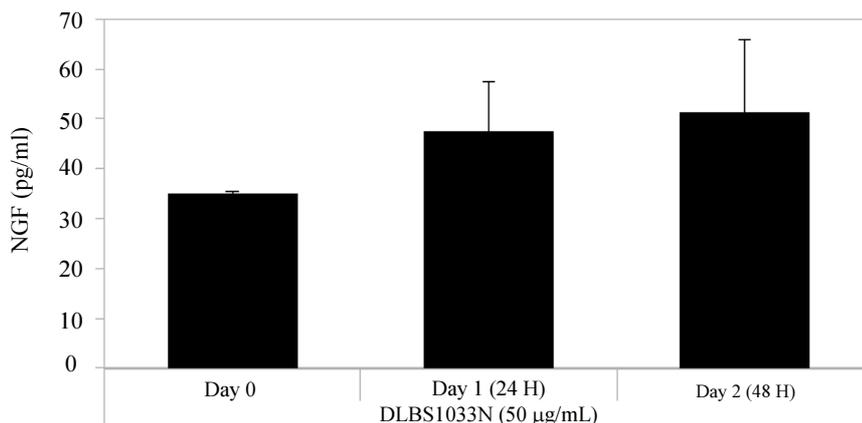


Fig. 5: NGF protein in cells media after treated with DLBS1033N, DLBS1033N induced NGF protein secretion in a time-dependent manner. RSC96 cell was treated with 50 µg mL⁻¹ DLBS1033N up to 48 h. The protein sample was isolated using RIPA buffer. NGF protein was detected using ELISA kit

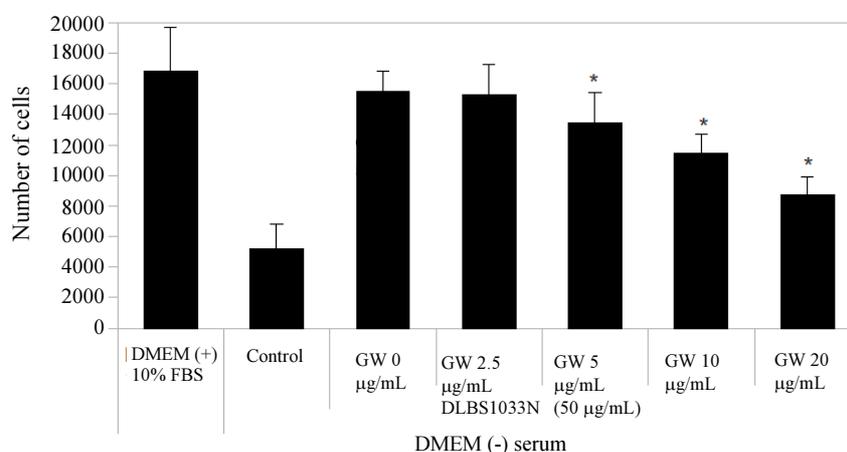


Fig. 6: GW441756 inhibit RSC96 cells growth, GW441756 inhibit cell growth by DLBS1033N. RSC96 cells were treated with/without various concentration of GW441756 ranging from 0 to 20 µg mL⁻¹ for 48 h. Values are expressed as means ± S.E. of eight independent experiments (*p<0.05, compared with GW441756 0 µg mL⁻¹)

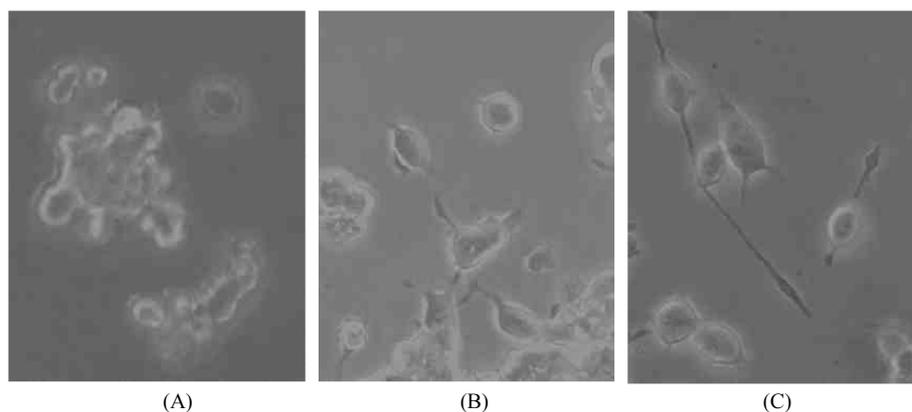


Fig. 7: DLBS1033N induces neurite outgrowth on PC12, The PC12 cell line was plated on 12-well plate coated with collagen at least 6 h. The PC12 cell line was treated for 72 h. (A) PC12 cell without any treatments. (B) PC12 cell with 0.1 ng mL⁻¹ NGF. (C) PC12 cell with 50 µg mL⁻¹ DLBS1033N

Table 2: Fold change of genes involved in both PI3K and MAPK pathways

PI3K gene	Fold change (48H) Sample		
	Control	DLBS1033N (50 µg mL ⁻¹)	Std. Deviation
PI3K	1	2.43	0.93
Akt	1	1.5	0.22
BAD	1	0.51	0.24
BCL-2	1	2.12	0.93
BCL-XI	1	1.34	0.18
MAPK gene			
MAPK	1	0.53	0.09
CREB	1	0.75	0.60
C-FOS	1	0.18	0.07
C-MYC	1	0.54	0.08

DLBS1033N Induces Neurite Outgrowth

Neurotrophin NGF was found to induce neurite outgrowth in nerve cell models PC-12 (ATCC® CRL1721™) cell line (Smit *et al.*, 2003; Greene *et al.*, 1987). The results showed that DLBS1033N could induce neurite growth in PC-12 cell line (Fig. 7). There was no neurite outgrowth detected on sample without treatments. Sample with 0.1 ng mL⁻¹ NGF experienced neurite outgrowth (Fig. 7B) and sample treated with DLBS1033N 50 µg mL⁻¹ experienced higher neurite outgrowth (Fig. 7C).

DLBS1033N Up-Regulated PI3K Related Genes

In order to find the mechanism activated by DLBS1033N, several gene expression from PI3K and MAPK pathways were investigated. DLBS1033N action on growth and survival is likely done via NGF-PI3K pathway (Table 2). Several gene expressions on MAPK pathway was tend to be down-regulated. DLBS1033N at 50 µg mL⁻¹ upregulated PI3K gene expressions for 2.43 fold compared to the control, then followed by the upregulation of the Akt gene expressions for 1.5 fold compared to the control. The BAD gene expressions were downregulated to 0.51 fold compared to the control. BCL-2 and BCL-xL were up-regulated by 2.12 and 1.34 fold, respectively, compared to the control.

Discussion

Proteins have diverse functions in living organisms, such as catalyzing biochemical reaction, transporting molecule and forming receptors and channels. With these diverse functions, proteins can be used for therapy. Therapeutic proteins have several advantages compared to small molecule drugs which include better specific actions and less adverse effects. Therapeutic proteins can also provide novel function or activity. Therapeutic proteins are mostly in the form of recombinant proteins. However, some therapeutic proteins can be obtained from their native sources (Leader *et al.*, 2008; Carter,

2011; Dmitrov, 2012). DLBS1033N is a protein fraction obtained from a natural resource, which is earthworm *Lumbricus rubellus*. In this study, DLBS1033N functions on Schwann cells exhibited its potential to be a therapeutic protein.

Schwann cells are one of the important factors in peripheral nerve regeneration process. Schwann cells help the nerve regeneration with its proliferation and differentiation process (Bunge, 1993; 1994; Ide, 1996). In this study, DLBS1033N was found to induce Schwann cells growth and survival. The ischemic factor can induce nerve injury. Weinberg *et al.* (2001) showed that people with critical limb ischemia can develop peripheral neuropathy. In this study, the investigation was focused on the ischemic factor. Two conditions were conducted in this study, which consisted of free serum condition and free serum with the minimum O₂ condition to mimick ischemic conditions. The observation showed that Schwann cells treated with DLBS1033N in free serum condition was resulted in higher cells population compared to the control, which was observed in 48 and 72 h of treatments. In free serum with minimum O₂ condition, Schwann cells treated with DLBS1033N for 24 h also showed higher cells population compared to the control.

In two different conditions, it was observed that DLBS1033N increased Schwann cells growth and survival. These results are indicated by the better survival of the Schwann cells at ischemic conditions with DLBS1033N treatment. FACS analysis also showed that samples treated with DLBS1033N have higher detected S phase compared to the control at 24, 36 and 48 h of treatments. It indicated that DLBS1033N increased cell proliferation activity. Kobayashi *et al.* (2012) and Jonsson *et al.* (2013) revealed that Schwann cell proliferation is a crucial step in nerve regeneration after ischemic nerve injury. Therefore, DLBS1033N actions on Schwann cells growth and survival are likely to exert several advantages in nerve regeneration process after ischemic injury.

The release of neurotrophins is also one of the Schwann cells activity on nerve regeneration process (Bunge, 1994). Neurotrophins are important regulators of neuronal survival, development, function and plasticity (Huang and Reichardt, 2001). Neurotrophins that have been characterized in mammals were NGF, BDNF, NT-3 and NT-4. Each neurotrophin has its own specific receptor (Hallbook, 1999). In this study, the effect of DLBS1033N on NGF and BDNF gene expressions were investigated. Other growth factors such as FGF and GDNF gene expressions were also observed. The experiments showed that DLBS1033N upregulated NGF gene expressions. The study was then continued at the protein level and found that NGF protein was also increased in a time-dependent manner. These two results indicated that growth and survival activities on Schwann cells were related to NGF expressions. Receptor inhibition assay was conducted to confirm the results. Neurotrophin NGF binds to TrkA receptors which can be inhibited by GW441756 (Jung *et al.*, 2008; Jung and Kim, 2008; Takemoto *et al.*, 2015). In inhibition study, the proliferation of DLBS1033N-treated Schwann cells were inhibited in a dose-dependent manner following GW441756 administrations. This result indicates that the growth and survival activities were induced by DLBS1033N which was also related to NGF expressions.

Based on growth-survival and NGF assays, it appeared that DLBS1033N was able to promote growth-survival of Schwann cells with NGF induction activity. DLBS1033N showed the potential to be a therapeutic protein for nerve regenerations. Earthworm extract is usually used for its lumbrokinase activity. Some studies, however, have revealed that earthworm extract can be used in nerve regeneration treatments. Chang *et al.* (2011a; 2011b) reported that Dilong can promote Schwann cells proliferation and migration. Dilong is consisted of earthworm *Pheretima aspergillum*. The earthworm species is different from *Lumbricus rubellus* which was used to prepare DLBS1033N. According to study by Chang *et al.* (2011a), Dilong was found to promote Schwann cell proliferation via IGF activation. The result is different to the current study which demonstrated the activity of DLBS1033N to promote Schwann cell proliferation via NGF induction. However, IGF expression was not further investigated in this study. Further studies may be needed to observe the effect of DLBS1033N on IGF expressions. Other study using earthworm *Pheretima aspergillum* was done by Chen *et al.* (2010). According to their study, *Pheretima aspergillum* water extract induced peripheral nerve regeneration *in vitro* and *in vivo*. Their *in vitro* study using PC12 cells revealed that *Pheretima aspergillum* water extract promoted neurite outgrowth in PC12 cells. In this study, DLBS1033N was also used in neurite outgrowth analysis. Experiment using PC12

cells line showed that the neurites outgrowth were detected in DLBS1033N-treated cells. This result indicates that DLBS1033N can promote neurites outgrowth on nerve cell models. In this study, it was indicated that DLBS1033N is likely not only work on Schwann cells but also on nerve cells model. Therefore, DLBS1033N is potential to be used as a therapeutic protein for peripheral nerve regenerations.

When NGF binds to TrkA receptor, it can activate several pathways such as PI3K and MAPK. Those two pathways are related to cells survival. Neurotrophins bind as dimers to p75 and Trk family members. Trk receptor dimerization leads to trans-autophosphorylation and activation of intracellular signaling cascades which increases PI3K and Akt activities. It also leads to the increased activity of MAPK (Huang and Reichardt, 2001; Yuan and Yankner, 2000; Chao, 2003).

In this study, several genes expressions related to PI3K and MAPK pathways were investigated. The result showed that PI3K pathway was more likely to be affected. Several genes that have been observed in PI3K pathway were PI3K, Akt, BAD, BCL-2 and BCL-xL. The investigation showed that DLBS1033N up-regulated PI3K gene expressions. Activation of PI3K is essential for the survival of many populations of neurons. Activation of PI3K will be followed by Akt activation. Several biological functions for cell survival were controlled and regulated by Akt (Datta *et al.*, 1999). Akt gene expressions in this study was also up-regulated. The other PI3K pathway genes observed were BAD, BCL-2 and BCL-xL. BAD plays a role in promoting cell death, while BCL-2 and BCL-xL promote cell survival. BAD is one of the Akt substrates. It is a BCL-2 family member that promotes apoptosis by binding to BCL-xL, which in the absence of binding will further inhibit the proapoptotic activity of Bax (Datta *et al.*, 1999; 1997). In this study, BAD gene expressions were downregulated. Therefore, chances for cells to experience apoptosis are less. Lesser chance for cells undergoing apoptosis is convinced with the upregulation of BCL-2 and BCL-xL gene expressions. BCL-2 and BCL-xL are BCL-2 family members that promote cell survival whereas other member such as Bax and BAD promote cell death. In the presence of survival factors, the activity of prosurvival BCL-2 family members prevails over the activity of prodeath BCL-2 family members (Datta *et al.*, 1999).

Activation of Trk receptor also increases MAPK activity. These in turn influence transcriptional events, such as the induction of cyclic AMP-Response Element Binding (CREB) transcription factor. CREB affects the cell cycles, neurite outgrowth and synaptic plasticity (Huang and Reichardt, 2001). MAPK gene expressions was down-regulated, which further also caused the downregulation of CREB gene expressions. The other MAPK pathway related genes are C-FOS and C-MYC.

MAPK is also known as ERK, one of the MAPK families. ERK is known to be an intracellular check point for cellular mitogenesis. ERK activation can induce immediate early gene C-FOS. The ERK can link G0/G1 mitogenic signals to the immediate early response. C-MYC was also activated following the activation of ERK. C-MYC is a DNA binding protein which is involved in transcriptional control of gene expression and has been known to be essential in cell proliferation (Zhang and Liu, 2002). In this study, C-FOS and C-MYC were downregulated. However, the Schwann cells were grown and survived. Therefore, there might be other pathways that lead the Schwann cells to grow. Further study of DLBS1033N on MAPK pathway might be needed.

Conclusion

In summary, DLBS1033N was found to promote the growth and survival of Schwann cells in free serum and free serum-minimum O₂ conditions. DLBS1033N was also able to induce NGF expressions. These two activities play important roles in peripheral nerve regenerations. In addition to the effect of DLBS1033N in Schwann cells, DLBS1033N also exhibited an activity in inducing neurite outgrowth on nerve cell model PC12 cell lines. These activities on Schwann cells and PC12 cell lines support DLBS1033N as a potential therapeutic protein candidate for peripheral nerve regenerations.

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Author's Contributions

Agung Heru Karsono: was responsible for the design, execution of the experimentation and analytical evaluation of the data and contributed to the writing of manuscript.

Raymond R. Tjandrawinata: was responsible for the design, execution of analytical evaluation and contributed to the writing of manuscript.

Maggy T. Suhartono: was responsible for the execution of analytical evaluation and contributed to the writing of manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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