Reports of Biochemistry & Molecular Biology
Vol.11, No.1, Apr 2022

Original article

www.RBMB.net

Melatonin Induced Schwann Cell Proliferation and Dedifferentiation Through NF-κB, FAK-Dependent but Src-Independent Pathways

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Abstract

Background: Peripheral nerve injury (PNI) is a common condition that compromises motor and sensory functions. Peripheral nerves are known to have regenerative capability and the pineal hormone, melatonin, is known to aid nerve regeneration. However, the role of Schwann cells and the pathways involved remain unclear. Thus, the aim of this study is to identify the effects of melatonin on Schwann cell proliferation, dedifferentiation, and the involvement of nuclear factor kappa light chain enhancer of activated B cells (NF-κB), focal adhesion kinase (FAK) and proto-oncogene tyrosine-protein kinase, Src pathways in this process.
Methods: Schwann cells was treated with melatonin and its proliferation and dedifferentiation were identified using MTT assay and immunofluorescence staining for SRY (sex determining region Y)-box 2 (SOX2). Next, the protein expressions of NF-κB, FAK and Src pathways were identified by Western blot.
Results: MTT results confirmed increased proliferation of Schwann cells with melatonin treatment, and it was highest at 10 μM melatonin. Immunofluorescent staining revealed an increase in the green fluorescence staining for SOX2 in melatonin-treated cells, showing enhanced dedifferentiation. Western blot assay revealed melatonin increased phospho-NF-κB (PNF-κB), IKK-α, FAK (D2R2E), phospho-FAK (Tyr 576/577 and Tyr 397) protein expressions as compared with control. However, Src (32G6), Lyn (C13F9), Fyn, Csk (C74C1) protein expressions were not increased as compared with control.
Conclusions: Melatonin promotes Schwann cell proliferation and dedifferentiation via NF-κB, FAK-dependent but Src-independent pathways.

Keywords: Dedifferentiation, Melatonin, Peripheral nerve injury, Proliferation, Schwann cells.

Introduction

Peripheral nerve injury (PNI) are mainly caused by road and workplace accidents (1). In the peripheral nervous system (PNS), neurons are primarily supported by a type of glial cell known as Schwann cell (2,3). In the absence of axonal interaction, mature Schwann cells can dedifferentiate into their immature form and later differentiate in the presence of axonal interaction and signalling (3). They are not directly involved in signal transmission between neurons but they perform neuronal axon myelination or ensheathment, guidance of axonal growth and removal of cellular debris (3). Following a neuronal injury, various types of molecular and cellular changes takes place, such as interference of retrograde signals flow and depolarization (4). Numerous types of neurotrophic factors, transcription factors, cytokines, growth factors, adhesion molecules, and structural elements are involved in this complex process. For example, the inhibition of Ras/Raf/MEK

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Received: 25 Apr, 2021; Accepted: 15 Aug, 2021

pathway expression upon axonal injury decreases the survival of neurons (2,4).

Melatonin, N-acetyl-5-methoxytryptamine, is a hormone secreted by the pineal gland in response to darkness and has been identified as the sleep hormone. Melatonin has showed potential treatment for various types of diseases, such as cancer, arterial hypertension, diabetes, irritable bowel disease, glaucoma, and macular degeneration (5). Melatonin plays an important role in nerve regeneration and functional recovery following PNI. It is proven that melatonin acts as an antioxidant and neuroprotectant by preventing oxidative stress, myelin breakdown and axonal damage at the injury site (6–9). Melatonin is also known to decreases fibroblast proliferation and collagen accumulation at the injury site by downregulating transforming growth factor (TGF)-β1 and basic fibroblast growth factor (bFGF), thus inhibiting neuroma formation (10,11). Although the effects of melatonin on nerve regeneration have been vastly studied, the optimum dose of melatonin in PNI treatment is yet to be identified. Till date, it is known that to improve PNI outcomes, melatonin must be present in supraphysiological level in the blood (12).

Multiple studies have been carried out to understand the correlation between melatonin and recovery after PNI. A study done on pinealectomised rats has shown that the presence of melatonin leads to a formation of significantly smaller neuroma and lesser adhesion following a nerve transection. The exogenous melatonin treatment also resulted in greater number of axons and lesser connective tissue formation at the proximal transected nerve (13). These changes make it easier for the proximal regenerating nerve to reach its target, thus leading to a functional recovery. Additionally, melatonin has been found to enhance Schwann cell proliferation and improve nerve recovery both *in vivo* and *in vitro*. Melatonin functions through the MT_1 receptor dependent extracellular signal-regulated protein kinase (ERK)1/2 pathway (14). The increased expression of SOX2 is correlated to oral mucosal carcinogenesis which is speculated to be important marker in cancer stemness (15). SOX2 expression is upregulated in immature Schwann cells after PNI event (16). NF-κB pathway is involved in many diseases and neuron regenerative process (17). Based on the literature, FAK and Src pathways are involved in Schwann cell proliferation and differentiation (18). In our study, we study the involvement of SOX2 and NF-κB in Schwann cells regeneration upon melatonin treatment.

Although the importance of Schwann cell in nerve recovery has been studied, minimal number of studies have been done to look at the molecular mechanism by which melatonin affects Schwann cell proliferation and dedifferentiation and the related signalling pathways. Thus, this study aims to evaluate if melatonin promotes Schwann cell dedifferentiation and proliferation through SOX2, NF-κB, FAK, and Src pathways. This information will provide a better understanding on how melatonin aids nerve recovery and the potential use of melatonin as a treatment for PNI in the future.

Materials and Methods

Materials

Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide tetrazolium (MTT), 98% absolute ethanol, protease inhibitor, and phosphatase inhibitor were purchased from (Sigma Aldrich, US.A.). Melatonin was purchased from (Calbiochem, U.S.A.). Trypsin–ethylenediaminetetraacetic acid (EDTA) was purchased from (Gibco, UK). Dimethyl sulfoxide (DMSO), Tween-20, dithiothreitol (DTT) and bovine serum albumin (BSA) were purchased from (Nacalai Tesque, Japan). Beta-actin (β-actin), NF-κB, PNF-κB, IKK-α, FAK (D2R2E), Tyr 925, Tyr 576/577, Tyr 397 (D20B1), Src (32G6), Fyn, Lyn (C13F9), Csk (C74C1), α-tubulin primary antibodies, and horseradish peroxidase-conjugated secondary antibodies were purchased from (Cell Signalling Technology, Danvers, MA). SOX2 primary antibody, and FITC conjugated

secondary antibody were purchased from (Abcam, UK). SuperSignal™ West Femto Maximum Sensitivity Substrate was purchased from (Thermo Fisher Scientific, Massachusetts, U.S.A.). Quick Start™ Bradford protein assay kit and bromophenol blue were purchased from (Bio-Rad, U.S.A.). Phosphate-buffered saline (PBS) was purchased from (Biobasic, Canada).

Cell culture and melatonin preparation

Schwann cell (RT4-D6P2T) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Schwann cells were cultured in DMEM and supplemented with 10% FBS at 37 ˚C in an incubator with 5% CO_2. Cells were trypsinized whenever the confluency of cells reach 80%. Stock melatonin was prepared at 10 mM using 98% absolute ethanol. The stock melatonin solution was then diluted to 500 µM using culture media and this was further diluted using culture media to prepare 0.5, 1, 5, 10 and 20 µM melatonin for treatment.

MTT assay

The proliferative effect of melatonin was measured in Schwann cells by MTT colorimetric assay. Schwann cells were seeded at 1×10^3 cells per well in 96-well plates and incubated for 24 h. Then, 0.5, 1, 5, 10 and 20 µM of melatonin were added and cultured for 24 hours. The cells were incubated with 20 µL of MTT (5 mg/ml) solution for 4 hours. Following medium removal, 80 µL of DMSO were added to each well and plates were gently shaken. The optical absorbance was measured at 570nm with 630nm as reference wavelength using Spectra Max3 (Molecular Devices, USA).

Immunofluorescence staining

The cells were seeded in 24-well plate at 1.5×10^4 cells/well. Later, treated with different concentrations of melatonin (0.5, 1, 5, 10 and 20 µM) for 24 hours. The cells were then fixed with 300 µL of 4% formalin formaldehyde for 15 minutes and blocked with 200 µL blocking buffer (5% FBS, 0.5% Tween-20, PBS) for one hour. Next, 200 µL of SOX2 primary antibody (1:50× dilution) was added and incubated overnight on a shaker at 4 °C. After incubating with primary antibodies overnight, 200 µL of FITC conjugated secondary antibody (1:100× dilution) was added and the cells were incubated for 3 h on a shaker, in the dark, at room temperature. Then, 200 µL Hoechst stain (diluted with PBS to 1µg/ml) was added and left for 15 min. Finally, 200 µL of PBS was added into each well and images were captured using inverted fluorescent microscope (Nikon Eclipse Ti, Japan). Fluorescent images were captured using GF and DAPI filter.

Western blot

The cells were seeded in 60-mm dish at a concentration of 1.5×10^4 cells/dish. Then, the cells were treated with different concentrations of melatonin (0.5, 1, 5, 10, and 20 µM) for 24 hours. Next, the cells were lysed with 200 µL of lysis buffer (0.0155 g/ml DTT, protease inhibitor (1:200), phosphatase inhibitor (1:100), 1× SDS). The cells were then heated at 96 °C for 5 min followed by centrifugation at 10,000 rpm for 10 min, at 4 °C. The protein concentration in the sample was determined using the Quick Start™ Bradford protein assay kit. Bromophenol blue was added to the protein samples (50 µg) to a final concentration of 0.01%. The protein samples were loaded into and separated by the SDS-PAGE gel. The separated proteins were then transferred to PVDF membrane. The membrane was then incubated with 6 mL blocking buffer (3% BSA diluted in TBST) for 2 h followed by incubation of NF-κB, PNF-κB, IKK-α, FAK (D2R2E), Tyr 925, Tyr 576/577, Tyr 397 (D20B1), Src (32G6), Fyn, Lyn (C13F9), Csk (C74C1), α-tubulin, and beta actin primary antibodies (1:1000× dilution) overnight at 4°C. Later, the blot was incubated with 6 mL horseradish peroxidase-conjugated anti-rabbit/mouse secondary antibody (1:10000× dilution) for 1 h. Finally, the blots were stained with chemiluminescent and the bands were imaged using ChemiDoc XRS$^+$ system (Bio-Rad). Densitometric analysis was performed using ImageLab Version 6.1.0 build

7 Standard Edition (Bio-Rad, Hercules, CA, USA) to scan the signals.

Statistical analysis

All data obtained from the MTT assay, immunofluorescent staining, and Western blot were expressed as mean±standard deviation. Student's t-test was done for data comparison, $p < 0.05$ indicates significant difference between the melatonin treated groups and the control.

Results

Effects of melatonin on Schwann cell proliferation

Cell proliferation was measured by MTT assay. Results showed that Schwann cells treated with melatonin had increased cell proliferation. Melatonin-treated cell proliferation was 10% - 20% greater as compared to the control. Melatonin concentration of 10 µM caused the highest percentage increase in Schwann cell proliferation (Fig. 1).

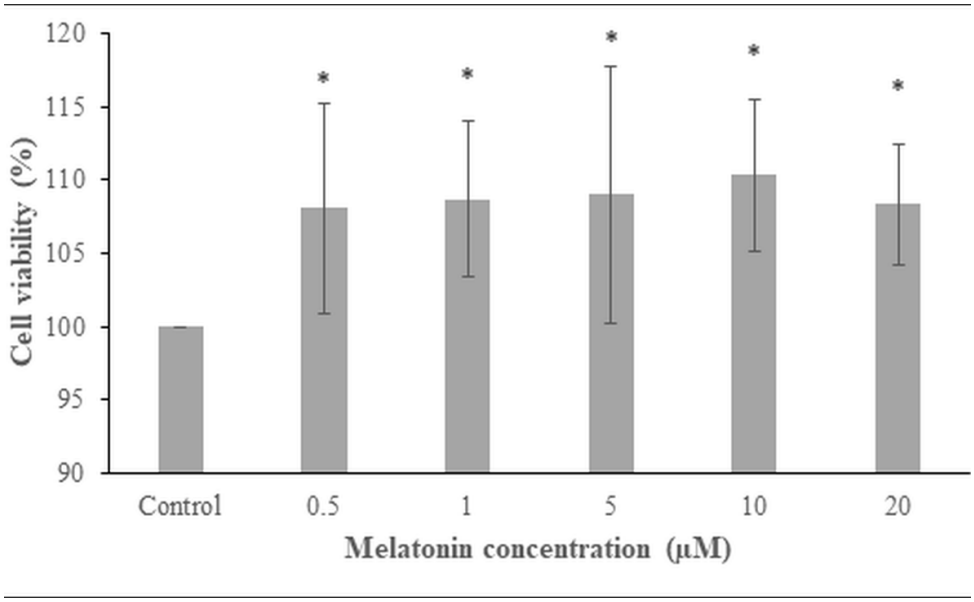

Fig. 1. Measurement of melatonin-treated and untreated Schwann cell proliferation using MTT assay. * indicates $p < 0.05$ when compare melatonin-treated Schwann cells with control.

Effects of melatonin on Schwann cell dedifferentiation

Immunofluorescence staining for SOX2 protein expression was performed at different melatonin concentrations (Fig. 2A). Results show that melatonin treated Schwann cells had increased green fluorescence, which reflects increased SOX2 protein expression. The number of cells expressing SOX2 protein (green fluorescence) was counted at each melatonin concentration, in different fields, with a total number of 100 cells. The average value was used to calculate the percentage increase of SOX2 protein expression in melatonin treated Schwann cells as compared to the control. The percentage of increase equals to treated groups divided by the untreated group. The bar chart shows that melatonin treated Schwann cells had a 24% - 29% increase in the percentage of SOX2 protein expression as compared to the control (Fig. 2B).

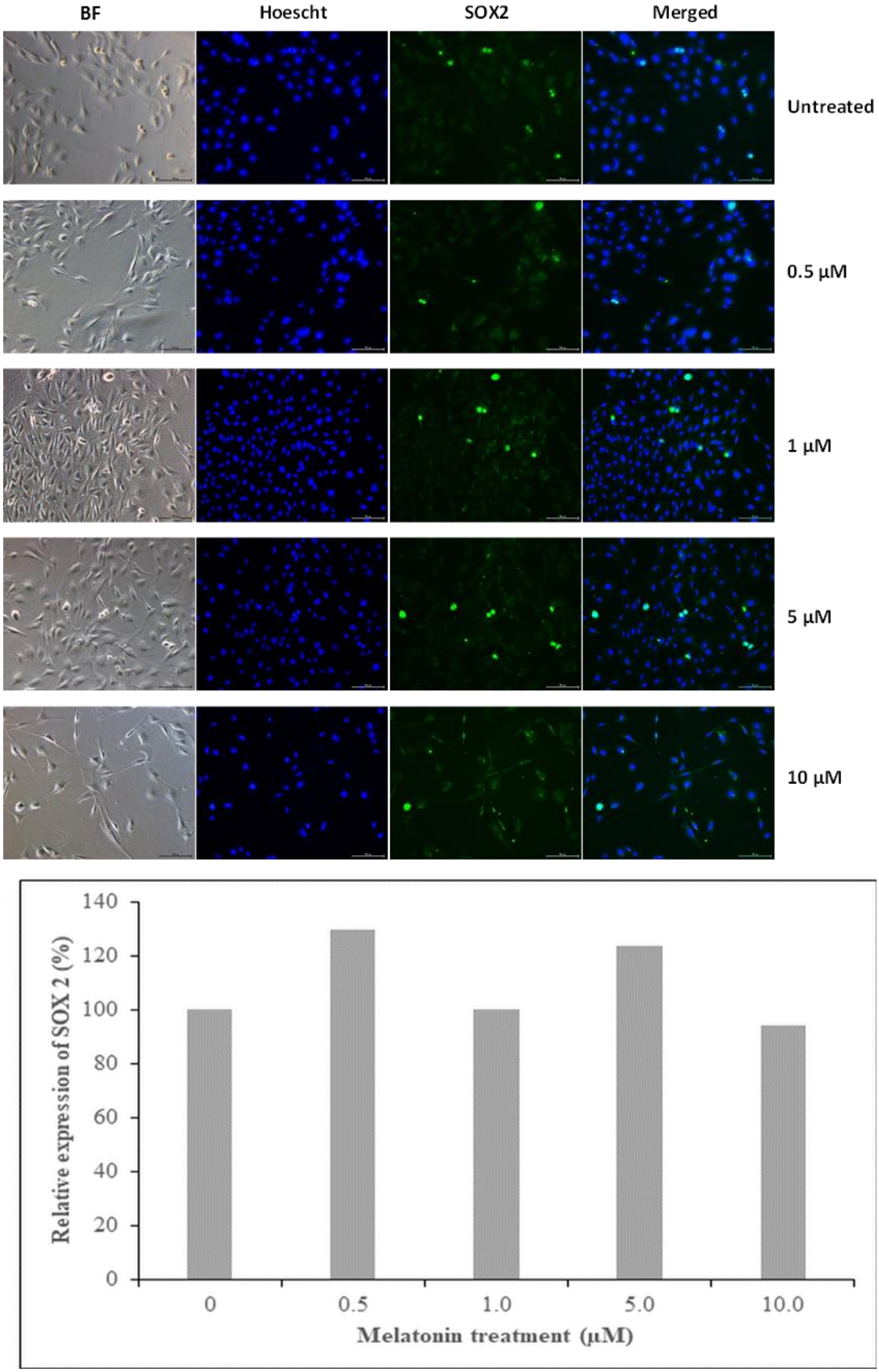

Fig. 2. Detection of SOX2 protein expression in melatonin-treated and untreated Schwann cells using immunofluorescence staining. After staining, A) melatonin-treated and untreated cells observed under light and fluorescence microscope, B) bar graph of SOX2 protein expression relative to control.

Effect of melatonin on NF-κB protein expressions in Schwann cells

NF-κB pathway consists of two mainstream proteins the NF-κB and PNF-κB and a downstream protein, IKK-α. Beta actin was used as a housekeeping protein. The results obtained show that melatonin treatment in Schwann cell increases PNF-κB and KK-α protein expressions as compared to the control (Fig. 3).

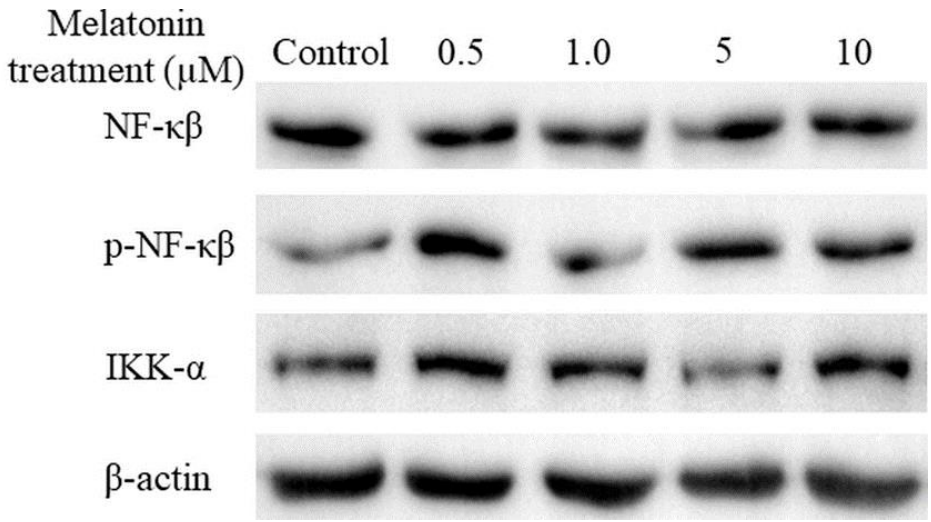

Fig. 3. Detection of NF-κB pathway protein expressions for melatonin-treated Schwann cells and control using Western blot assay.

Effect of melatonin on FAK pathway protein expressions in Schwann cells

The D2R2E is the original FAK protein while the Tyr 925, Tyr 576/577 and Tyr 397 are phosphorylated FAK proteins of different sizes. Beta actin was used as a housekeeping protein. The results obtained show that melatonin treatment in Schwann cell increases FAK, FAK phosphorylated (Tyr 576/577 and Tyr 397) protein expressions as compared to the control (Fig. 4). However, the expression of FAK phosphorylated (Tyr 925) does not increase as compared with control (Fig. 4).

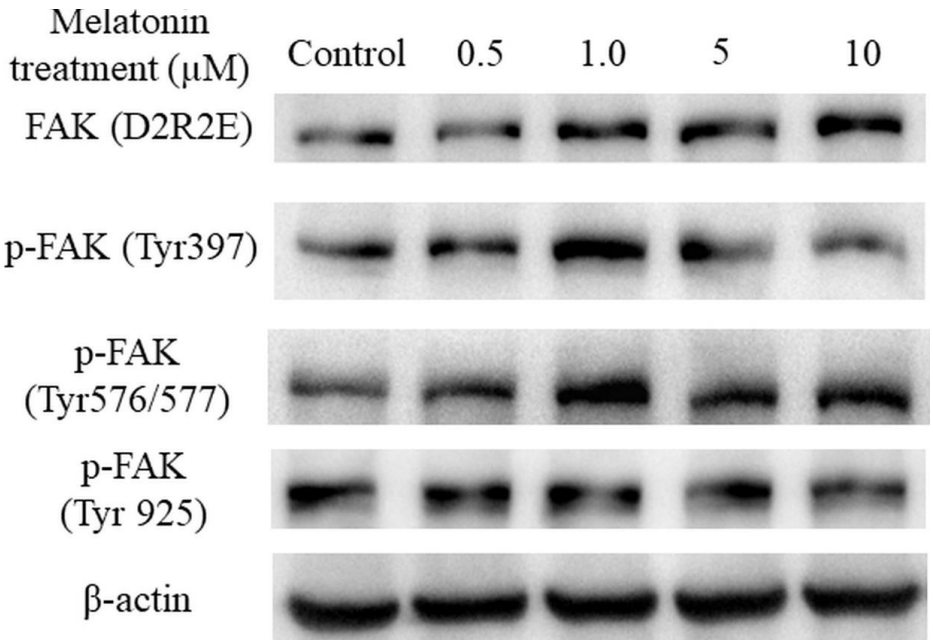

Fig. 4. Detection of FAK pathway protein expressions for melatonin-treated Schwann cells and control using Western blot assay.

Effect of melatonin on Src family protein expressions in Schwann cells

Src family proteins include Src (32G6), Fyn, Lyn (C13F9) and Csk. Our results showed that Src (32G6), Lyn (C13F9), Fyn, Csk protein expressions do not increase in the melatonin treated groups as compared to the control (Fig. 5). Beta actin was used as a housekeeping protein.

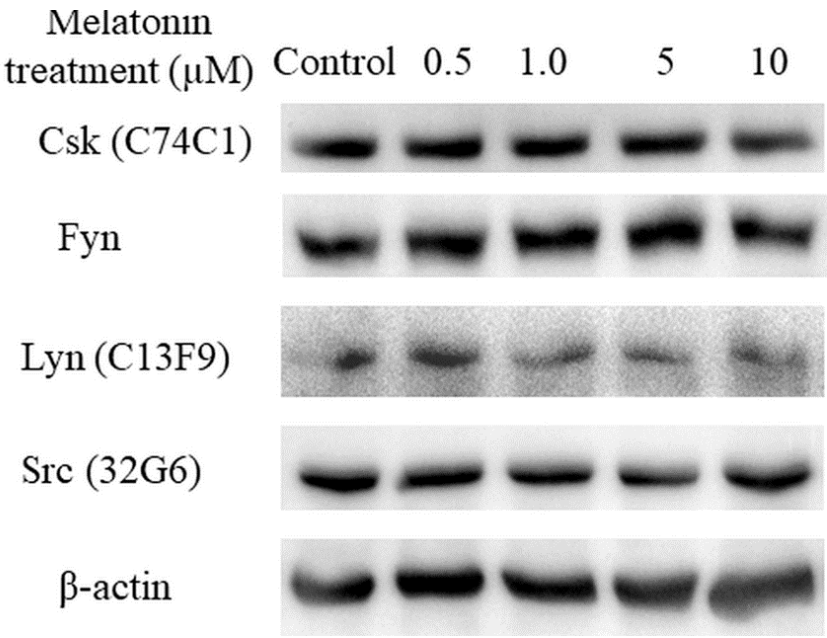

Fig. 5. Detection of Src family protein expressions for melatonin-treated Schwann cells and control using Western blot assay.

Discussion

In our study, the melatonin increased proliferation and induced dedifferentiation of Schwann cells via expression of SOX2 protein, NF-κB, and FAK pathways activation. However, involvement of Src pathway was not detected in melatonin treated-Schwann cells.

We tested the effects of melatonin on Schwann cell proliferation at different concentrations. Our results show that melatonin increases Schwann cell proliferation at 0.5, 1, 5, 10 and 20 μM as compared to the control (Fig. 1). These observations are supported by a previous study that demonstrated an increase in Schwann cell proliferation both *in vivo* and *in vitro* when treated with melatonin and its positive correlation with target reinnervation. This proliferative effects of melatonin was believed to be regulated through the MT_1-dependent ERK1/2 signalling pathway (14). In addition, our results showed that Schwann cells treated with 10 μM melatonin for 24 hours had the highest proliferation and we noted a decrease in percentage of cell viability at a melatonin concentration above 10 μM. This observation was in line with the previous study that revealed the effects of melatonin on Schwann cell proliferation was not directly related to the dose. This was reflected by their results that were more significant at 1 mg/kg compared to 10 mg/kg *in vivo* studies (14). These findings may be due to the toxicity of melatonin on the cells at higher doses.

The transcription factors expressed by Schwann cells changes as they differentiate from neural crest cells to the mature form. SOX2 protein is a transcription factor expressed on the cell surface of Schwann cells at some stages (16). Following PNI, mature Schwann cells are known to dedifferentiate into the repair phenotype and SOX2 expression is upregulated. Thus, it is suggested

that SOX2 protein is a marker of immature Schwann cells (16,19). With this information, we tested the effects of different concentration of melatonin on Schwann cell dedifferentiation by immunofluorescent staining for SOX2 protein. Our results showed that the expression of SOX2 protein in the melatonin treated-Schwann cells were higher as compared to the control (Fig. 2). The increased SOX2 protein expression with melatonin treatment suggests that melatonin promotes Schwann cell dedifferentiation into the immature form. This SOX2 protein expression is crucial because following PNI, it plays a role in maintaining the Schwann cells in the immature state, increasing its proliferative response, inhibiting re-myelination, recruiting macrophages to injury site and supports cell sorting and migration to guide the regenerating axon. These roles are important for proper nerve regeneration and functional recovery (16,19-22).

Since we were able to demonstrate that melatonin induces Schwann cell proliferation and dedifferentiation, next we studied the involvement of NF-κB pathway in this process. NF-κB pathway is known to play a major role in Schwann cell differentiation and peripheral nerve myelination (23). It is also known that NF-κB is not involved in Schwann cell survival and proliferation but is important for axonal sorting. Following PNI, the cytokines at the injury site activates NF-κB pathway which plays a crucial role in nerve regeneration and remyelination. Inhibition of NF-κB causes a temporary delay in axonal regeneration and remyelination which is later enhanced to reach normal levels. This is possibly due to the increased regenerative capability after injury, but a decrease in the rate of regeneration, thus directly decreasing myelination rate. NF-κB pathway consists of two mainstream proteins the NF-κB and p-NF-κB and a downstream protein, IKK-α (24). Our results show that Schwann cells treated with melatonin have an increasing trend of p-NF-κB and IKK-α protein expressions as compared to the control. The increase of p-NF-κB and IKK-α protein expressions can help to improve nerve recovery and suggests the beneficial role of melatonin in PNI treatment.

We studied the involvement of FAK pathway in melatonin induced Schwann cell proliferation and dedifferentiation. Studies showed that FAK pathway regulates Schwann cell proliferation, differentiation, and myelination. The interaction of laminin and integrin which are located between Schwann cells and ECM are responsible for FAK phosphorylation. FAK are also known to be further phosphorylated by Src to cause greater activation (25). During development, FAK promotes Schwann cells spreading and contractility on the fragmented basal lamina, thus enhancing Schwann cell proliferation and inhibiting premature differentiation into myelinating phenotype. High number of Schwann cells are crucial for radial sorting and Schwann cell differentiation for myelination (26). A study showed that myelination by FAK-negative Schwann cells was severely impaired during development and it was concluded that the failure of FAK-null Schwann cells to myelinate axons was due to the lack of Schwann cells (27). In consistence with these studies, our results show that Schwann cells treated with melatonin have an increasing trend of FAK (D2R2E) and phospho-FAK (Tyr 576/577 and Tyr 397) protein expressions as compared to the control. The increase of these protein expressions can help nerve development and suggests the beneficial role of melatonin in developmental neuropathies.

We studied the involvement of Src family proteins in melatonin induced Schwann cell proliferation and dedifferentiation. The Src family proteins are non-receptor tyrosine kinases consisting of c-Src, c-Yes, c-Fgr, Lck, Blk, Hck, Fyn and Lyn proteins, each having various role in cell proliferation, differentiation, survival, and apoptosis. The Src family proteins are usually present in inactive form, activated in association with cell response and rapidly degraded or inactivated following appropriate response (28). Src family proteins are expressed during peripheral nerve development and axonal

regeneration. Both the Src and FAK pathways are involved in Schwann cell development induced by the progesterone metabolite allopregnanolone (29). Activated c-Src and c-Fyn are both present in intact peripheral nerve and following injury the c-Src levels increase while c-Fyn levels are variable in the distal site of injury (30). The upregulated active Src expression was also noted in Schwann cells distal to the injury site. Src activation is crucial for Schwann cells adhesion, spreading and growth which are all crucial for axonal regeneration (31). In addition, Lyn and Fyn are known to initiate peripheral myelination and Schwann cell differentiation through activation of p38 MAPK, Akt and ERK pathway and Krox-20 expression (30). In this study, our results show that melatonin-treated Schwann cells do not increase Src (32G6) Lyn (C13F9), Fyn, Csk (C74C1) protein expressions as compared to the control. Thus, melatonin did not induce Schwann cell proliferation and dedifferentiation through this pathway.

This study proves that melatonin enhances Schwann cell proliferation and dedifferentiation through the involvement of SOX2, NF-κB, and FAK pathways but not Src pathway. In conclusion, melatonin treatment plays a beneficial role in nerve regeneration following PNI. In the future, it has a potential to be used as a treatment option following PNI to enhance functional recovery.

Acknowledgements

This research was funded by International Medical University, grant number is BMS I-2020 (18).

All the authors reported that there is no conflict of interest.

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