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The effect of calcium supplementation on attachment and growth of Schwann cells in DMEM D-Valine media

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ABSTRACT

Schwann cell (SC), a vital component of the peripheral nervous system, functions in supporting neurons through the production of the myelin sheath. Current approaches are utilising in vitro cultured Schwann cells in transplantation therapy for demyelinating diseases. Despite the therapeutic potential of SCs, it is challenging to maintain the growth and proliferation of these cells in vitro due to several factors, including supplementation of calcium ion (Ca^{2+}) for cell attachment. Thus, this study aimed to determine the effect of Ca^{2+} supplementation on SCs growth in vitro. SCs were isolated from rat's sciatic nerve and were cultured in the following media: i) DMEM D-valine + 1.8 mM Ca^{2+}; or ii) DMEM D-valine + 5.0 mM Ca^{2+}. Cell attachment was observed in both groups. Findings of this study demonstrated that at day 20 of culture, 90% of cells attachment was observed in a culture dish containing DMEM D-valine + 1.8 mM Ca^{2+}. However, limited attachment was seen in DMEM D-valine + 5.0 mM Ca^{2+} within the same period of observation. The cultured cells were subjected for immunocytochemistry analysis with Schwann cells protein marker, s100b. The cultured cells for both groups were positive for the expression of S100b. In conclusion, a high concentration of Ca^{2+} prevented cell attachment and cellular growth by interfering with the state of cell electrolyte, thus resulted in the damage of the cell plasma membrane.

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INTRODUCTION

Schwann cells (SCs), the main glial cell in the peripheral nervous system (PNS), are important for the production of the myelin sheath surrounding the axon. Due to this function, SCs are utilised as a repair strategy for treating diseases of the peripheral and central nervous system (CNS) (Kocsis and Waxman, 2007; Woodhoo *et al.,* 2007). Investigation in the regulation of cell phenotype and cell proliferation using cultured SCs often require a large population of cells as well as a stable microenvironment for cells to grow in an in vitro setting.

Previously, numerous methods have been introduced to isolate and culture SCs, such as the isolation of SCs from sciatic nerve (Evans, 2002; Wei *et al.,* 2009; Kaewkhaw, Scutt and Haycock, 2012; Tao, 2013; Weiss *et al.,* 2018), differentiation of mesenchymal stem cells into Schwann cells-like-cells (Hassan *et al.,* 2012a; Hassan *et al.,* 2012b; Sulong *et al.,* 2014), and differentiation of adipose stem cells into Schwann cells-like-cells (Jiang *et al.,* 2008; Wei *et al.,* 2010; Younesi *et al.,* 2015; Sowa *et al.,* 2016). In spite of this, obtaining a highly purified culture of SCs is often difficult due to several factors, i.e. cells harvesting technique, digestion method, and nutrition content of the culture media. In addition, the slow growth of SCs causes an overgrowth of the fibroblast cells in

culture and death of SCs (Brockes *et al.,* 1979). Therefore, it is vital for these SCs to adhere quickly to the culture surface to improve the number of SCs in culture.

Calcium, an inorganic salt present in the culture medium, is one of the important factors that prevent cell attachment on the culture surface that will eventually activate cellular apoptotic cascade. Previous studies have reported that nerve injury-induced over-secretion of calcium ion (Ca^{2+}) in the cell, leading to structural damage and cell death (Orrenius and Nicotera, 1994; Ziv and Spira, 1995; Sattler *et al.,* 1996; Arundine and Tymianski, 2003). Therefore, this study was aimed to evaluate the effects of Ca^{2+} supplementation on SCs attachment and growth in DMEM D-valine media to improve the culturing methods for SCs.

METHODS

Project Approval

The usage of animals for this project has been approved by Universiti Kuala Lumpur, Institute of Medical Science Technology (UniKL MESTECH)'s Animal Ethics Committee (FYP/AEC/MESTECH-UniKL/2017/008/JULY-2017-DEC-2017).

Collection of Rat's Sciatic Nerves and SC Culture

Sciatic nerves used in this study were obtained from Sprague-Dawley rats aged eight to ten weeks. To harvest the sciatic nerve, the rat was positioned ventral side down with stretched lower limbs. Surrounding skin above the gluteus maximus muscles was removed and approximately 1.5 cm length of sciatic nerves was bilaterally dissected. This fresh sciatic nerve was subjected to enzymatic dissociation at 1.0% (w/v) collagenase solution for 60 minutes at 37°C, 5% CO_2 with agitation. SCs isolated from the sciatic nerve was selected by plastic adhesion. The cells were cultured in two conditions medium; (i) DMEM D-valine + 1.8 mM Ca^{2+} and (ii) DMEM D-valine + 5.0 mM Ca^{2+}. Both culture media were supplemented with 10% FBS and 5 µM of forskolin and cultured for 20 days before they were ready for in vitro evaluation. Prior to cell seeding, the culture dish for both culture media was coated with poly-L-lysine and laminin.

Evaluation of Cell Attachment with Phase Contrast Microscope

Cell attachment was evaluated in both culture media with a phase contrast microscope (CX41 Phase Contrast Olympus Microscope, Japan) up until day 20. The culture was not physically disturbed until approximately day five and observation was conducted very gently, as the force generated by movements of the culture medium will disturb early SCs attachment. One millilitre of culture medium was added on day seven. 50% of used culture medium was removed and changed with a new culture medium on day 11 to allow better observation of cells attachment. Cultured cells were allowed to confluence and on day 20, cell viability was calculated with Trypan blue (0.4%) method.

Immunocytochemistry Analysis of SCs with SC Protein Marker

Cultured SCs were fixed with 4% (v/v) formaldehyde solution for 20 minutes. The cells were further incubated with primary antibody (polyclonal anti-S100b) in 1% (w/v) bovine serum albumin (BSA) at 4°C overnight. The following day, cells were incubated with FITC anti-mouse IgG secondary antibody (1:100 in 1% (w/v) BSA) at room temperature for 60 minutes. Nuclei of the cells were counter-stained with 300 nM DAPI for 20 minutes. The cells were finally examined using a fluorescence microscope (Nikon Eclipse E600, Japan).

RESULTS

Evaluation of Cell Attachment

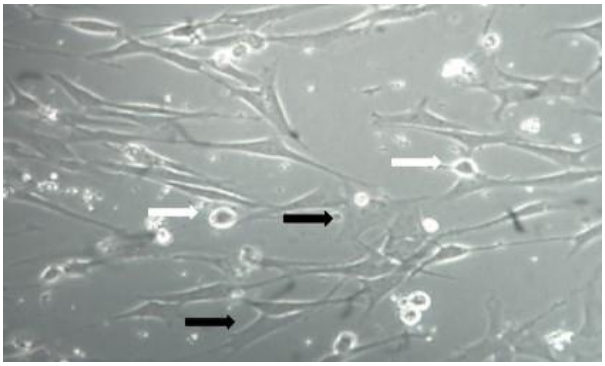

Figure 1: Phase contrast micrographs of Fibroblast cells (black arrows) and Schwann cells (white arrows) attachment to the culture dish on day 8 in vitro

Microscopic observation using phase-contrast microscopy demonstrated cell attachments to the poly-L-lysine and laminin pre-coated plastic surface of the culture dish. In the DMEM D-valine + 1.8 mM Ca^{2+} contained media, two different types of cell were observed, the SCs and fibroblast cells. The SCs appeared as bipolar or tripolar elongated morphology with a small nucleus (low nucleus-to-cytoplasmic ratio), while fibroblasts exhibited more flattened polymorphic shape with a large nucleus (Figure 1). A higher proportion of monolayer cells attached at the bottom of the culture dish, mainly the SCs and some fibroblasts were observed on day 12 when using the media containing DMEM D-valine + 1.8 mM Ca^{2+} (Figure 2a). Cell attachments were also observed to be intensively increased on day 20 of culture forming a 'classical' parallel swirling alignment of dendritic

processes (Figure 2b). Limited fibroblast attachment was noted due to the presence of D-valine in the culture media which specifically functions to enhance SCs proliferation.

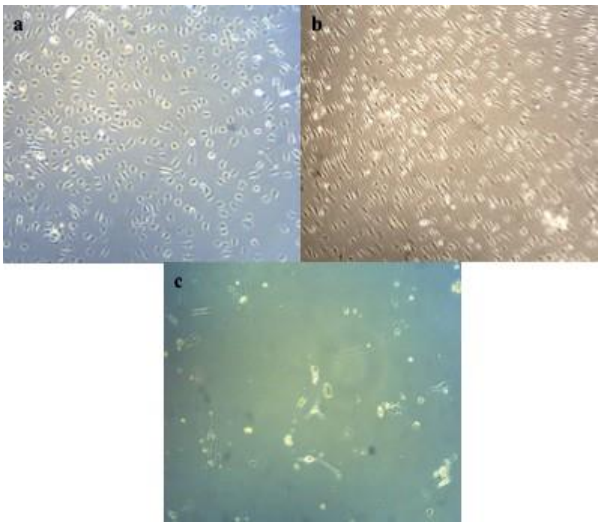

Figure 2: DMEM D-valine + 1.8 mM Ca2+ contained media demonstrated Schwann cell's attachment at day 12 (a) and 20 (b). In contrast, only few cell attachments were observed in DMEM D-valine + 5.0 mM Ca2+ (c) (magnification: 10X)

To substantiate the inhibitory effect of high Ca^{2+} level in a media on cell attachment, DMEM D-valine + 5.0 mM Ca^{2+} was used with the same mitogenic factors retained in the culture medium. Under this condition, limited cell attachment (SCs or fibroblast) can be detected within the same observation timeline (Figure 2c). Although mitogenic factors for both types of cells were present in the culture medium, they did not assist in cell proliferation due to the inhibition of primary attachment. Hence, appropriate Ca^{2+} level in the culture medium is essential to support the primary cell attachment onto the culture dish.

To evaluate the survival of the cultured cell, cell viability was calculated on day 20 for both culture conditions. Cell cultured in DMEM D-valine + 5.0 mM Ca^{2+} only showed 15% viability as compared to cells that were cultured in DMEM D-valine + 1.8 mM Ca^{2+} demonstrated 90% viability.

Immunocytochemistry of SCs

SCs in primary cultures (passage 0, P0) can be identified by immunolabeling of the phenotypic marker S100b. All cells with a typical bipolar or tripolar elongated morphology should stain positively for this marker. SCs culture in this study demonstrated localization of S100b in the nucleus and cytoplasm. In contrast, no expression of the S100b protein in the fibroblast population (cells with flattened polymorphic shape and a large nucleus) was detected, cells only labelled positively for the nuclear marker DAPI (Figure 3).

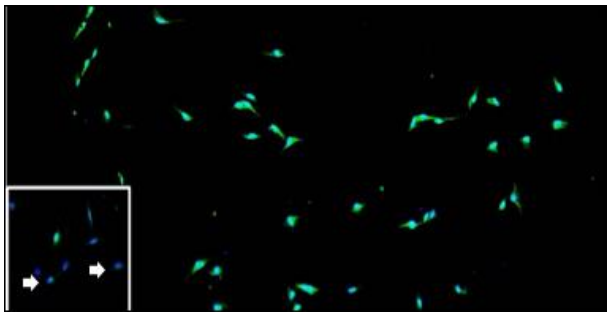

Figure 3: Immunocytochemistry evaluation of cultured cells showed positivity towards S100b (insert: Fibroblasts stained with DAPI (arrows))

DISCUSSION

Attachment of primary cells on the culture dish surface has a large impact on the growth and survivability of these cells. SCs is classified as adherent cells and require a surface, such as culture dish coated with extracellular matrix (such as laminin and poly-L-lysine used in this study) to enhance adhesion and attachment properties. This event will also provide other signals that are essential for growth and differentiation. Following the attachment, cell movement and proliferation will be triggered within the in vitro culture control environment.

For PNS, SCs are the most essential glial cells and its presence is crucial during nerve regeneration. To date, the development of alternative therapeutic approach in treating peripheral nerve injuries is actively researched around the world. One of it is the use of autologous Schwann cells for transplantation to the injured nerve, which involves the isolation of patient's SCs and in vitro expansion of the cells number in the laboratory with addition or supplementation of mitogens using cell culture techniques. This method is essential to obtain an abundant source of autologous graft materials from a small biopsy sample for use in cells transplantation. However, SCs are known to be very difficult to culture in vitro. Many approaches introduced to culture SCs resulted in low yield due to its slow proliferation rate. Fibroblast contamination, on the other hand, worsened the condition further. It is believed that the primary attachment of SCs to the culture dish for the first five days without any physical or mechanical disturbances are highly crucial for this type of cell and will determine the survivability in vitro.

Results of this study demonstrated poor attachment and growth of SCs following exposure to high Ca^{2+} concentration. We speculated that membrane cell damage has occurred due to the

osmosis effect from high Ca^{2+} level consequently leading to low cell attachment in the culture. Therefore, intracellular Ca^{2+} homeostasis must be maintained at all times to avoid membrane damage to the SCs which will subsequently lead to overgrowth of fibroblast. Fibroblast overgrowth can occur because of its ability to proliferate very quickly and is able to suppress SCs growth.

Similarly, in vivo studies reported a boost in Ca^{2+} concentration in neuronal axoplasm following peripheral nerve injury, directly affects the survival and growth of surrounding cells including SCs (Orrenius and Nicotera, 1994; Zhang and David, 2016; Yang *et al.,* 2017). Under normal condition, low basal expression of Ca^{2+} is required to maintain ionic gradients in the axoplasm. This is to prevent the activation of apoptotic cascade due to an elevation of Ca^{2+} influx that impairs cellular functions of neurons as well as surrounding cells such as the SCs (Schlaepfer and Bunge, 1973). Ca^{2+} has the largest gradient across the plasma membrane of all living cells compared to other ions, and they are biologically very active. As a consequence, to cellular injury, the high Ca^{2+} influx will cause irreversible plasma membrane damage, leading to a large gradient of Ca^{2+} thereby triggering the accumulation of Ca^{2+} intracellularly. This will further activate a type of cell death cascade called necrosis. Calculation of cell viability verified the occurrence of cell necrosis. Cell cultured in high Ca^{2+} concentration only showed 15% viability. This finding suggested that optimum Ca^{2+} concentration in the culture medium is crucial not only to enhance cell attachment but also to maintain cell's survival in vitro.

CONCLUSION

Our study concluded that SCs attachment, survival and growth are very sensitive and are negatively affected by exposure to high Ca^{2+} levels. Appropriate Ca^{2+} concentration in SCs culture media is extremely critical to maintain balanced homeostasis so as to avoid lethal plasma membrane damage and other consequences which may transform a living cell into a necrotic one.

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