ARTIGO ORIGINAL

Acta Med Port 2005; 18: 323-328

INTRACELLULAR Ca^{2+} CONCENTRATION IN THE N1E-115 NEURONAL CELL LINE AND ITS USE FOR PERIPHERIC NERVE REGENERATION

J. M. RODRIGUES, A. L. LUÍS, J. V. LOBATO, M. V. PINTO, A. FAUSTINO, N. SOORAJ HUSSAIN, M. A. LOPES, A. P. VELOSO, M. FREITAS, S. GEUNA, J. D. SANTOS, A. C. MAURÍCIO

Centro de Estudos de Ciência Animal. Instituto de Ciências e Tecnologias Agrárias e Agro-Alimentares. Departamento de Clínicas Veterinárias, Instituto de Ciências Biomédicas de Abel Salazar. Universidade do Porto. Hospital S. João. Faculdade de Motricidade Humana, Universidade Técnica de Lisboa. Faculdade de Engenharia da Universidade do Porto. Porto. Dipartimento di Scienze Cliniche e Biologiche, Università di Torino. Italy. Departamento de Saúde Pública, Braga. Centro Hospitalar de Vila Nova de Gaia, Gaia. Portugal. Portugal

SUMMARY

Entubulation repair of peripheral nerve injuries has a lengthy history. Several experimental and clinical studies have explored the effectiveness of many biodegradable and non-degradable tubes with or without addition of molecules and cells. The main objective of the present study was to develop an economical and also an easy way for culturing a neural cell line which is capable of growing, differentiating and producing locally nerve growth factors, that are otherwise extremely expensive, inside 90 PLA / 10 PLG nerve guides. For this purpose the authors have chosen the N1E-115 cell line, a clone of cells derived from mouse neuroblastoma C-1300 with the perspective of using this differentiated cellular system to cover the inside of 90 PLA / 10 PLG nerve guides placed to bridge a nerve gap of 10 mm in the rat sciatic nerve experimental model. The N1E-115 cells proliferate in normal culture medium but undergo neuronal differentiation in response to DMSO. Upon induction of differentiation, proliferation of N1E-115 cells ceases, extensive neurite outgrowth is observed and the membranes become highly excitable. While it is known that Ca^{2+} serves as an important intracellular signal for cellular various processes, such as growth and differentiation, be toxic to cells and be involved in the triggering of events leading to excitotoxic cell death in neurons. The $[Ca^{2+}]_i$ in non-differentiated N1E-115 cells and after distinct periods of differentiation, have been determined by the epifluorescence technique using the Fura-2-AM probe. The results of this quantitative assessment, revealed that N1E-115 cells which undergo neuronal differentiation for 48 hours in the presence of 1.5% DMSO are best qualified to be used to cover the interior of the nerve guides since the $[Ca^{2+}]_i$ was not found to be elevated indicating thus that the onset the cell death processes was not occurred.

Key Words: *N1E-115 cells, Nerve Regeneration, PLGA, Intracellular Calcium Concentration, Epifluorescence Technique.*

Recebido para publicação: 14 de Dezembro de 2004

RESUMO

CONCENTRAÇÃO INTRACELULAR DE Ca^{2+} DAS CÉLULAS NEURONAIS N1E-115 E A SUA UTILIZAÇÃO NA REGENERAÇÃO DO NERVO PERIFÉRICO

A utilização de tubos-guia na reparação de nervos periféricos apresenta uma longa história. Vários estudos experimentais e ensaios clínicos testaram a utilização de tubos-guia construídos a partir de biomateriais biodegradáveis e não biodegradáveis, na presença de moléculas e de diversas células. O principal objectivo deste trabalho consiste no desenvolvimento de uma linha celular económica, fácil de manter em cultura e que seja capaz de crescer, diferenciar-se e de produzir os factores neurotróficos e neurotrópicos localmente, quando utilizados para forrar os tubos-guia de 90 PLA / 10 PLG. Para isso, foi escolhida a linha celular N1E-115, derivada de um clone celular de neuroblastomas de rato C-1300, para ser utilizada como sistema celular diferenciado no revestimento interior dos tubos-guia de 90 PLA / 10 PLG. Estes tubos-guia são utilizados para reconstruir uma solução de continuidade de 10 mm no nervo ciático no modelo experimental que é o rato. As células neuronais N1E-115 proliferam facilmente em meio de cultura normal mas iniciam a sua diferenciação em resposta à presença de DMSO. Após indução da diferenciação, a proliferação das células N1E-115 é interrompida, surgem longos prolongamentos citoplasmáticos e as suas membranas plasmáticas tornam-se altamente excitáveis. É conhecido, de uma série de trabalhos publicados, que o Ca^{2+} é um 2º mensageiro intracelular muito importante numa série de processos e eventos celulares, nomeadamente crescimento e diferenciação. É igualmente tóxico para as células a partir de determinada concentração intracelular, estando igualmente envolvido no despoletar de uma série de eventos intracelulares que conduzem à morte celular, nomeadamente de neurónios. A $[Ca^{2+}]_i$ nas células N1E-115 indiferenciadas e após vários períodos de tempo de diferenciação, na presença de DMSO foi determinada, recorrendo à técnica de epifluorescência e utilizando como sonda fluorescente a molécula anfifílica Fura-2AM. Os resultados desta determinação quantitativa levaram-nos a concluir que as células N1E-115 diferenciadas durante 48 horas na presença de 1.5% de DMSO são as mais qualificadas para serem utilizadas no revestimento dos tubos-guia, uma vez que a $[Ca^{2+}]_i$ não está elevada para iniciar o processo de apoptose ou morte celular. Ao fim destas 48 horas apresentam alterações morfológicas características de diferenciação, as mitoses foram suspensas e são capazes de produzir factores neurotróficos e neurotrópicos no local de reconstrução do nervo periférico à semelhança de células neuronais normais.

Palavras-chave: *Células N1E-115, regeneração nervosa, PLGA, concentração de cálcio intracelular, técnica de epifluorescência.*

INTRODUCTION

Peripheral nerve regeneration is a complex biological phenomenon and nerve injury is a serious health problem for Society today. The injuries of the peripheral nerve are the result of trauma, disease or surgical procedures that require section of peripheral nerves to gain access to the surgical site[1]. The nervous system comprises two cell types: neurons and neuroglia. Glial cells are found to be more abundant than neurons, and unlike neurons, which cannot undergo mitosis, glial cells have some capacity for cell division. Although neurons cannot divide by mitosis, they can regenerate a severed portion of their axon and / or sprout new processes under certain conditions[2]. The aim of the use of first nerve guides was to offer to regenerating axons optimal conditions where the influence of external non-cellular and humoral factors have been minimized, and where only cells and tissue elements normally occurring in a peripheral nerve trunk would influence the regeneration process[3]. Since that time, the end of the Eighteen Century, a number of different materials have been explored for use in aiding nerve regeneration. Poly(esters), such as poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and poly(lactic-co-glycolic acid) (PLGA)[4,5] were early candidates used in testing because of their availability, ease

of process, and approval by the FDA[6]. Also, the role of neurotrophic factors in neural regeneration has been the focus of extensive research[2]. The influence of these factors in neural development, survival, outgrowth, and branching has been explored on various levels, from molecular interactions to macroscopic tissue responses. One family of neurotrophic factors, the neurotrophins, has been heavily investigated in nerve regeneration studies, including the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin – 3 (NT-3), and neurotrophin – 4/5 (NT- 4/5)[7]. Neurotrophic factors promote a variety of neural responses: survival and outgrowth of the motor and sensory nerve fibers, spinal cord and peripheral nerve regeneration[2,8]. However, *in vivo* responses to neurotrophic factors can vary due to the method of their delivering. Therefore, the development and use of highly controlled delivery devices are required for the study of these extremely complex systems. For that reason, N1E-115 cell line established from neuroblastoma, that undergo neuronal differentiation in response to DMSO, cAMP, or serum withdrawal[9-12] might be an important cellular system to locally produce and deliver these neurotrophic factors. Upon induction of differentiation, proliferation of N1E-115 cells ceases, extensive neurite outgrowth is observed and the membranes become highly excitable[9,10]. During differentiation, cyclin-dependent kinase (cdk) activities decline and phosphorylation of the retinoblastoma gene product (pRb) is lost, leading to the appearance of a pRb-containing E2F DNA-binding complex. The molecular mechanism by which pRb inhibits cell proliferation is becoming increasingly clear. pRb inhibits the activity of proteins that function as inducers of DNA synthesis[13]. Ca^{2+} serves as an important intracellular signal for cellular processes such as growth and differentiation. Free Ca^{2+} levels within neural cells control many essential neural functions including neurotransmitter release, membrane conductance, nerve fibber excitability, coupling between neuronal cells, and axonal transport. Although regulation of the intracellular calcium concentration ($[Ca^{2+}]_i$) is important for normal cell functioning, its deregulation has been linked to cellular pathologies and cell death[14]. Deregulation in $[Ca^{2+}]_i$ can be toxic to cells and is involved in the triggering of events leading to excitotoxic cell death in neurons, through the activation of calpain, phospholipases and endonucleases[15]. For example, especially in neurons from the central nervous system (CNS), anoxia results in uncontrolled increases in $[Ca^{2+}]_i$ that can eventually lead to cell death[16]. Because of the importance of $[Ca^{2+}]_i$ in neuronal health and disease, a relatively simple cell model system, one where $[Ca^{2+}]_i$ regulation can be studied fairly easily, is desirable.

The purpose of this study was to test a non-expensive and easy method to culture neural cell line capable of producing locally, nerve growth factors (that are extremely expensive) and of growing these cells on 90 PLA / 10 PLG tubular nerve guides, in order to use them to promote nerve regeneration across a peripheral nerve gap. To correlate the neuronal cells' ability to promote nerve regeneration across a gap through their differentiation grade and survival capacity, the $[Ca^{2+}]_i$ of non-differentiated N1E-115 cells was determined by the epifluorescence technique using the Fura-2AM probe[17,18], and after 48 and 72 hours of differentiation. The measurement of $[Ca^{2+}]_i$ permitted to determine an ideal period of differentiation, when the N1E-115 cells presented already the morphological characteristics of neuronal cells and at the same time, the death process was not initiated by the $[Ca^{2+}]_i$ modifications.

MATERIALS AND METHODS

Cell culture

N1E-115 is a clone of cells derived from mouse neuroblastoma C-1300[19] and retains numerous biochemical, physiological, and morphological properties of differentiated neuronal cells in culture[20]. N1E-115 neuronal cells were cultured in Petri dishes (around 2 x 106 cells) on 25-mm-diameter glass poly-l-lysine-coated coverslips or on 90 PLA / 10 PLG fragments at 37ºC, 5% CO_2 in an humidified atmosphere with 90% Dulbecco's Modified Eagle's Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco) (Maintenance Medium). The culture medium was changed every 48 hours and the Petri dishes were observed daily in an inverted microscope. The cells were passed or were supplied with differentiation medium once they reached approximately 80% confluence, mostly 48 hours after plating. The differentiation medium was composed by 96% DMEM supplemented with 2.5 % of FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin and 1.5% of DMSO (Differentiation Medium). The cells were used before differentiation and after 48 and 72 hours of differentiation in the presence of the low serum and 1.5% DMSO medium (Differentiation Medium).

Epifluorescence Technique

Intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured in Fura-2-loaded cells by using dual wavelength spectrofluorometry as previously described[17].

Ca^{2+} indicator Fura-2/AM loading

N1E-115 mouse neuroblastoma cells were loaded with

Ca^{2+} indicator by incubation in 2.5 mM Fura-2 acetixymethyl ester (Fura-2/AM, Molecular Probes) and 0.03% Pluronic (Molecular Probe) in a Ringer Solution with the following composition: 121 mM NaCl, 5.4 mM KCl, 9 mM D-glucose, 1.5 mM $MgCl_2$, 1.8 mM $CaCl_2$, 6 mM $NaHCO_3$, and 25 mM HEPES, with a pH of 7.4; at 37ºC in darkness for 60 minutes.

Measurement of Intracellular Ca^{2+} in N1E-115

After loading Fura-2/AM, N1E-115 cells were washed in Ringer Solution (121 mM NaCl, 5.4 mM KCl, 9 mM D-glucose, 1.5 mM $MgCl_2$, 1.8mM $CaCl_2$, 6 mM $NaHCO_3$, and 25 mM HEPES, with a pH 7.4). The glass coverslips with adhering N1E-115 cells were transferred to a glass chamber containing 100 µl of the Ringer Solution. The chamber was placed in a well on the stage of an epifluorescence microscope (Zeiss, Germany). Fluorescence measurements were performed in each individual cell. The emitted fluorescence intensities at 510 nm were acquired by computer software, which registered the number of photons emitted per second, during 30 s for each 340 nm and 380 nm excitation wavelengths. The $[Ca^{2+}]_i$ was estimated from the ratio equation described by Grynkiewiez and colleagues[21]. For determination background fluorescence, cells were incubated in 2.5 mM 4--br-A23186 (Molecular Probe) and 10 mM $MnCl_2$ in 100 µl of Ringer Solution at room temperature in darkness for 10 minutes. The $[Ca^{2+}]_i$ measurements considered for these results were the ones which background signal was inferior to 20% of the total emitted fluorescence.

90 PLA / 10 PLG Preparation

Poly (dl-lactide-co-glycolide) copolymers with ratio of 90 PLA/ 10 PLG were obtained from their cyclic dimers, i.e. dl-lactide and glycolide. For this study, non-woven constructs were used to prepare tube guides with the following dimensions: 20 mm long, internal diameter of 1.5 mm and thickness wall of 1.5mm. These fully synthetic non-woven structures are very flexible biologically safe materials and can sustain the compressive forces due to body movement after implantation. They have also some degree of porosity to allow for influx of low molecular nutrients required for nerve regeneration. These tube guides of PLGA were expected to degrade in several weeks to body metabolites (lactic and glycolic acid) by hydrolysis of ester bonds. N1E-115 neuronal cells (around 2 x 106 cells) were cultured on 25-mm--diameter 90 PLA / 10 PLG fragments at 37ºC, 5% CO_2 in a humidified atmosphere with Maintenance Medium. These N1E-115 cells were differentiated in the presence of the low serum and 1.5% DMSO medium (Differentiation Medium).

Statistics

All data were presented as mean ± SEM, N is the number of cells where the $[Ca^{2+}]_i$ was measured by the epifluorescence technique. All statistical tests were Student's t test[22]. The given P values correspond to errors of the second kind ($P < 0.05$).

RESULTS

90 PLA /10 PLG

90 PLA / 10 PLG nerve tube-guides presented porosity, thus, the N1E-115 cells sub-cultured on fragments of this biomaterial, get an easy access to the culture medium components. These cells could be differentiated in the presence of 1.5 % DMSO and low serum concentration (Differentiation Medium), in a similar way the N1E--115 cells sub-cultured on glass coverslips pre-treated with poli-l-lisine.

N1E-115 Neuronal Cell Culture

The N1E-115 cells cultured on glass coverslips pre--treated with poli-l-lisine or on 90 PLA / 10 PLG fragments when supplied with Maintenance Medium achieved 80% of confluence in 48 hours. The differentiation has been induced, by supplying the cells with Differentiation Medium, which is basically a low serum medium with 1.5% of DMSO. Figure 1 shows images obtained in an inverted microscope (Zeiss, Germany) with an amplification of 100x. This sequence of pictures presents N1E-115 cells (around 2 x 106 cells) sub-cultured on glass coverslips pre-treated with poli-l-lisine, with 12, 48, 60 and 72 hours of differentiation in the presence of 1.5% DMSO and low serum medium (Differentiation Medium). After 48 hours of the differentiation process, these cells exhibited cytoplasmatic prolongations and the cell growth and mitosis was inhibited. The N1E-115 cells in the presence of this Differentiation Medium acquired the morphological and physiological characteristics of normal neural cells. In Figure 2, pictures of N1E--115 cells (around 2 x 106 cells) sub-cultured on 90 PLA / 10 PLG fragments are presented, with a magnification of 100x, obtained with an optic microscope (Zeiss, Germany). The N1E-115 cells were submitted to a differentiation process like the N1E-115 cells described for Figure 1, for periods between 12 to 72 hours. After 48 hours in the presence of 1.5% of DMSO and low serum concentration these cells already exhibited extensive neurite outgrowth and mitosis has ceased.

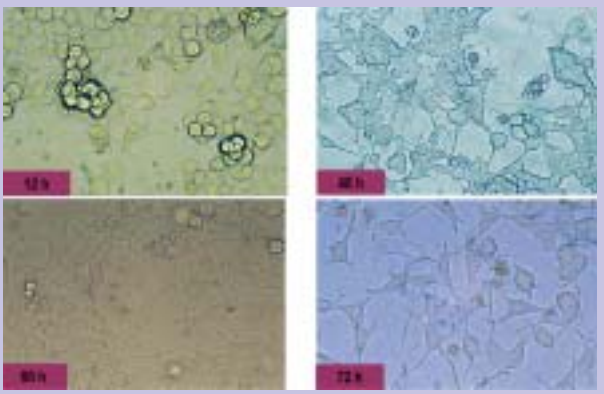

Fig. 1 - N1E-115 cells cultured on glass coverslips supplied with differentiation medium for periods between 12 to 72 hours. After 48 hours of differentiation in the presence of 1.5% DMSO these cells exhibited cytoplasmaticprolongations and the cell growth and mitosis was inhibited. Images obtained in an inverted microscope (Zeiss, Germany) with an amplification of 100x.

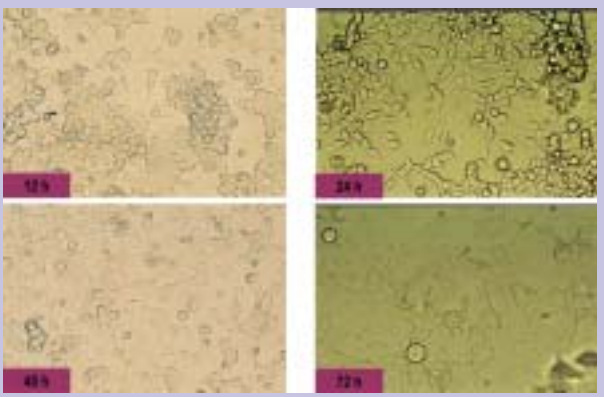

Fig. 2 - N1E-115 cells cultured on 90 PLA / 10 PLG fragments, supplied with differentiation medium for periods between 12 to 72 hours. After 48 hours of differentiation in the presence of 1.5% DMSO these cells exhibited cytoplasmaticprolongations and the cell growth and mitosis was inhibited. Images obtained in an optic microscope (Zeiss, Germany) with an amplification of 100x.

$[Ca^{2+}]_i$ determination

The $[Ca^{2+}]_i$ was measured by the epifluorescence technique using the Fura-2-AM probe[18]. The results presented in Table 1 are referred to measurements made in non-differentiated N1E-115 cells, after 48 and 72 hours of differentiation in the presence of 1.5% of DMSO, cultured on glass coverslips pre-treated with poli-l-lisine. The $[Ca^{2+}]_i$ of non-differentiated N1E-115 cells was 37.6 ± 3.9 nM (N = 24). The $[Ca^{2+}]_i$ of N1E-115 cells after 48 and 72 hours of differentiation was 50.8 ± 3.5 nM (N = 15) and 145.8 ± 9.4 nM (N = 15), respectively. The mean value of $[Ca^{2+}]_i$ determined in non--differentiated N1E-115 cells and in N1E115 cells after 48 hours of differentiation in the presence of 1.5% DMSO was not statistically different for $P < 0.05$. Also the mean value of $[Ca^{2+}]_i$ determined in N1E-115 cells after 48 hours of differentiation and in differentiated N1E-115 cells during 72 hours in the presence of 1.5% DMSO was statistically different for $P < 0.05$.

TABLE 1 - *$[Ca^{2+}]_i$ imeasured by the epifluorescence technique, using the Fura-2-AM probe in non -differentiated N1E-115 cells, after 48 and 72 hours of differentiation in the presence of 1.5% of DMSO (*). Results are presented as mean and standard error of the mean (SEM). N corresponds to the number of N1E-115 cells analyzed. All statistical tests were Student's t test.The P values given correspond to errors of the second kind ($P < 0.05$).*

Cell Type	N1E-115 not differentiated	N1E-115 With 48h differentiation*	N1E-115 with 72h differentiation*
$[Ca^{2+}]_i$ nM	37.6 ±3.9 N = 24	50.8 ±3.5 N = 15	145.8 ±9.4 N = 15

DISCUSSION

The purpose of this study was to develop a not expensive and easy procedure to culture a neural cell line capable of producing, locally, nerve growth factors that are otherwise extremely expensive. This differentiated cellular system will be used in further studies to cover the inside of PLGA nerve guide that will be placed in a nerve gap of 10 mm of the rat sciatic nerve experimental model. This procedure has also the advantage that the nerve growth factors are produced in physiological concentrations in the site of the regeneration process, a condition that is extremely difficult to accomplish by the exogenous administration. This neural cell line can be obtained in any Cell Culture Bank, and is very easy to maintain in culture at 37ºC, 5% CO_2 in a humidified atmosphere and reached approximately 80% confluence after 24-48 hours. N1E-115 cells proliferated in normal culture medium and could be submitted to neuronal differentiation in response to 1.5 % of DMSO and low serum concentration. The PLGA nerve tube-guides presented porosity permitting that the N1E-115 cells sub-cultured on these fragments had easy access to the culture medium components. These cells could be differentiated in the presence of 1.5 % DMSO and low serum concentration, in a way similar to N1E-115 cells sub-cultured on glass coverslips pre-treated with poli-l-lisine. After 48 hours of differentiation, the N1E-115 cells (adherent to glass coverslips previously treated with poli-l-lisine or to PLGA fragments) already exhibited cytoplasmatic prolongations and the cell growth and mitosis were inhibited. These results suggest that N1E-115 cells that undergo neuronal differentiation for 48 hours in the presence of 1.5% DMSO are best qualified to cover the interior of the tubular nerve guides since, in 48 hours of differentiation, these cells presented morphological

differentiation without a simultaneously increase of the $[Ca^{2+}]_i$ which leads to the onset cell death. Results obtain by Kopper and Adorante[23] indicated that the $[Ca^{2+}]_i$ in viable non-differentiated N1E-115 neuroblastoma cells was 52.9 ± 2.5 (N = 15). When these cells have started the death process, in the presence of extracellular Na+, the $[Ca^{2+}]$i rose to 105.3 ± 2.5 nM (N = 15) and in the absence of extracellular Na^+, the $[Ca^{2+}]_i$ rose to values even greater of 129.5 ± 15.4 nM (N = 8). These measurements have been performed in N1E-115 cells that were not submitted to the differentiation process. This peak of the $[Ca^{2+}]_i$ was not due to the a capacitive Ca^{2+} entry, but was the result of Ca^{2+} release from intracellular stores. However, Mathes and Thompson[24] did find evidence for a capacitive Ca^{2+} entry in DMSO--differentiated N1E-115 cells, so the $[Ca^{2+}]_i$ increase was superior, to values around 150 nM. It has been shown that the Ca^{2+}-gated K^+ channel in N1E-115 cells, responsible for the capacitive Ca^{2+} entry, is only expressed following the differentiation process by DMSO[23,25]. These results are in agreement with our $[Ca^{2+}]_i$ measurements performed in non--differentiated and in differentiated N1E-115 cells.

On the basis of the results obtained in the present study, N1E-115 cells that will be employed to cover the interior walls of the PLGA nerve guides, will be firstly differentiated in the presence of 1.5% DMSO. After 48 hours of *in vitro* differentiation, the tubular guides (inside covered by this cellular system) will be used to reconstruct a nerve gap of 10 mm by a surgical procedure. This approach will permit the local production of nerve growth factors, in a physiological concentration, which might facilitate regeneration process.

CONCLUSIONS

N1E-115 cells that undergo neuronal differentiation for 48 hours in the presence of 1.5% DMSO are best qualified to cover the interior of PLGA tubular nerve guides.

ACKNOWLEDGMENTS

The authors would like to gratefully acknowledge the valuable support by José Manuel Correia Costa, from Laboratório de Parasitologia, Instituto Nacional de Saúde Dr. Ricardo Jorge and Artur Varejão from Universidade de Trás-os-Montes e Alto Douro (UTAD). They would like to thank Pedro Costa for kindly provide the N1E-115 neuronal cells.

REFERENCES

1. BELKAS JS, SCHOICHET MS, Midha R: Peripheral nerve regeneration through guidance tubes. Neurological Research 2004; 26: 151 – 160.
2. SCHMIDT CE, LEACH JB: Neural Tissue Engineering: Strategies for Repair and Regeneration. Annu Rev Biomed Eng 2003; 5: 293 – 347.
3. LUNDBORG G: Nerve Injury and Repair. London: Churchill Livingstone 1988; pp: 149 – 195.
4. MOLANDER H, OLSSON Y, ENGKVIST O, BOWALD S, ERIKSSON I: Regeneration of peripheral nerve through a polyglatin tube. Muscle Nerve 1982; 5: 54 – 57.
5. NYILAS E, CHIU TH , SIDMAN RL , HENRY EW , BRUSHART TM, DIKKES P, MADISON R: Peripheral nerve repair with bioresorbable prosthesis. Trans. Am. Soc. Art. Intern. Organs 1983; 29: 307 – 313.
6. HUDSON TW, EVANS GR, SCHMIDT CE: Engineering strategies for peripheral nerve repair. Clin Plast Surg 1999; 26: 617 – 628.
7. LUNDBORG G, DAHLIN I, DANIELSEN N, ZHAO Q: Trophism, tropism and specificity in nerve regeneration. J Reconstr Microsurg 1994; 10: 345 – 354.
8. LONGO FM, HAYMAN EG, DAVIS GE, RUOSLAHTII E, ENGVALL E, MANTHORPE M, VARON S: Neurite-promoting factors and extracellular matrix components accumulating in vivo within nerve regeneration chambers. Brain Res; 1984; 309: 105 – 117.
9. KIMHY Y, SPECTOR I, BARAK Y, LITTAUER UZ: Maturation of neuroblastoma cells in the presence of dimethylsulfoxide. Proc Natl Acad Sci USA 1976; 73: 462 – 466.
10. DE LAAT SL, VAN DER SAAG PT: The plasma membrane as a regulatory site in growth and differentiation of neuroblastoma cells. In Rev Cyt 1982; 74: 1 – 53.
11. REAGAN LP, YE X, MIR R, DEPALO R, FLUHARTY J: Up--regulation of angiotensin II receptors by in vitro differentiation of murine N1E-115 neuroblastoma cells. Mol Pharmacol 1990; 38: 878 – 886.
12. LARCHER JC, BASSEVILLE M, VAYSIERRE JL, CORDEAU--LOSSOUARN L, CROIZAT B, GROS F: Growth inhibition of N1E--115 mouse neuroblastoma cells by c-myc or N-myc antisense oligodeoxynucleotides causes limited differentiation but is not coupled to neurite formation. Biochem Biophys Res Comun 1992; 185: 915 – 924.
13. KRANENBURG O, SCHARNHORST V, VAN DER EB AJ, ZANTEMA A: Inhibition of cyclin-dependent kinase activity triggers neuronal differentiation of mouse neuroblastoma cells. The Journal of Cell Biology 1995; 131(1): 227 – 234.
14. TRUMP BF, BEREZESKY IK: Calcium-mediated cell injury and cell death. FASEB J 1995; 9: 219 – 228.
15. SMITH KY, HALL SM: Peripheral demyelination and remyelination initiated by the calcium – selective ionophore ionomycin: in vivo observations. J Neurol Sci 1988; 83(1): 37 – 53.
16. STYS PK: Anoxic and ischemic injury of myelinated axons in CNS white matter: from mechanistic concepts to therapeutics. J Cereb Blood Flow Metab 1998; 18(1): 2 – 25.
17. FISHER S, DOMANSK L, ROLAND R: Muscarinic receptor regulation of cytoplasmatic calcium concentration in human SK-N-SH neuroblastoma cells: calcium requirements for phospholipase C activation. Mol Pharmacol 1989; 35: 195 – 204.
18. TSIEN R: Fluorescent probes of cell signalling. Ann Rev Neurosc 1989; 12: 227 – 242.
19. AMANO TER, NIRENBERG M: Neurotransmitter synthesis by neuroblastoma clones. Proc Natl Acad Sci USA 1972: 69: 258 – 263.
20. TUTTER J B, RICHELSON E: Pheytoin action on the excitable membrane of mouse neuroblastoma. J Pharmacol Exp Ther 1979; 211(3): 632 - 637.
21. GRYNKIEWICZ G, POENIE M, TSIEN RY: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985; 260: 3440 - 3450.
22. HALD A: Statistical Theory with Engineering Applications, pp. 1952; 391-394. John Wiley, New York.
23. KOPPER KL, ADORANTE JS: Regulation of intracellular calcium in N1E-115 neuroblastoma cells: the role of $Na+/Ca^{2+}$ exchange. Am J Physiol Cell Physiol 2002; 282: C1000 – C1008.
24. MATHES C, THOMPSON SH: The relationship between depletion of the intracellular Ca^{2+} stores and activation of Ca^{2+} currents by muscarinic receptors in neuroblastoma cells. J Gen Physiol 1995; 106: 975 – 993.
25. SPECTOR I: Electrophysiology of clonal nerve cell lines. In: Excitable Cells in Tissue Culture, edited by Nelson PG and Lieberman M. New York: Plenum, 1981; p 247 – 277.