GKDA Derg 2020;26(3):115-22
doi: 10.5222/GKDAD.2020.07078

Araştırma / *Research Article*

Neuroprotective Effects of Levosimendan on the NB2a Mouse Neuroblastoma Cell Culture

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Levosimendan'ın NB2a Fare Nöroblastoma Hücre Kültürü Üzerine Nöroprotektif Etkileri

Etik Kurul Onayı: Etik onay Manisa Celal Bayar Üniversitesi Tıp Etik Kurulu tarafından verildi (No: 10.05.2018-20.478.480).
Çıkar çatışması: Çıkar çatışmaları hiçbir yazarın bu makaleye katkıda bulunmasını engellemez.
Finansal destek: Bu çalışma, Celal Bayar Üniversitesi Bilimsel Araştırma Kurumu (2016/105) tarafından desteklenmiştir.

Ethics Committee Approval: Etik Onay Manisa Celal Bayar Üniversitesi Tıp Etik Kurulu tarafından verildi (No: 10.05.2018-20.478.480).
Conflict of interest: Conflicts of interest do not preclude any of the authors from contributing to this manuscript.
Funding: This work was supported by the Scientific Research Council of Celal Bayar University (2016/105).

Cite as: Öztürk T, Vural K, Aydemir I, Tuğlu Mİ. Levosimendan'ın NB2a fare nöroblastoma hücre kültürü üzerine nöroprotektif etkileri. GKDA Derg. 2020;26(3):115-22.

ABSTRACT

Objective: *Levosimendan (LVS) inhibits phosphodiestherase III and opens the ATP-sensitive K+ channels (KATP). It is cardioprotective due to its anti-oxidant, anti-inflammatuary, inotropic and vasodilatatory effects. The use of LVS is prefered in patients who have decreased ejection fraction during cardiac surgery. Therefore it has been reported that LVS protects neurons indirectly. Hovewer, its direct effects on neurons have not been known yet. We examined direct neuroprotective effects of LVS using neuroblastoma cell line of mouse origin (NB2a) in culture where drug interaction with cells occurs.*
Method: *To this end, the neuroprotective effects of LVS (0,1, 0,3, 1, 3, 10, 30 and 100 µM) have been tested for its direct toxic effects by TUNEL method for apoptosis and by MTT for cell viability. We also examined moderate and chronic toxic effect of LSV by measure neurite outgrowth.*
Results: *LVS didn't effect the cellular proliferation negatively. The number of apoptotic cells didn't differ from the number of control cells (p>0.05). Additionally, any moderate neurotoxic effects were not seen in all concentrations of NST which did not inhibit neurite outgrowth. Moreover, LVS at 1 µM concentration significantly increased the lengh of neurite showing clearly its neuroprotective and functional effects.*
Conclusion: *These direct neuroprotective effects of LVS in culture might be important for clinical use. LVS can be used securely in patients that have a risk for a brain injury due to the nature of disease, trauma or the procedure itself.*

Keywords: *neurotoxicity, in vitro, levosimendan, NB2a, MTT*

ÖZ

Amaç: *Levosimendan (LVS) Phosfodiestheraz III'ü inhibe eder ve ATP-duyarlı K+ kanallarını açar. Anti-oxidan, antiinflammatuar, inotropik ve vasodilatator etkileri ile kardiyoprotektiftir. LVS, kardiyak cerrahi sırasında düşük düşük ejeksiyon fraksiyonlu hastalarda kullanımı tercihedilir. Böylece nöronları indirekt olarak koruduğu bildirilmiştir. Ancak, nöronlar üzerine direk etkileri bilinmemektedir. Biz, fare kökenli nöroblastoma hücre dizinini kullanarak (NB2a) hücrelerle ilaç etkileşiminin olduğu kültürde LVS'nin direkt nöroprotektif etkilerini araştırdık.*
Yöntem: *Bu amaçla, LVS (0,1, 0,3, 1, 3, 10, 30 and 100 µM)'nın nöroprotektif etkileri, direkt toksik etkileri için appopitozis TUNEL metot ile ve hücre canlılığı için MTT ile test edildi. Aynı zamanda LSV'nın orta ve kronik toksik etkileri için nörit uzaması ölçüldü.*
Bulgular: *LVS hücre çoğalmasına negatif etki etmedi. Apopitotik hücrelerin sayısı kontrol hücrelerininkinden farklı değildi (p>0.05). Buna ek olarak, nörit inhibisyonunun görülmediği NST ile tüm konsantrasyonlarda orta derecede nörotoksik etki yoktu. 1 µM konsantrasyonda LVS, nöroprotektif ve fonksiyonel etkiyi açıkça gösteren nörit uzamasını anlamlı olarak artırdı.*
Sonuç: *LVS'nın kültürde direkt nöroprotektif etkileri klinik kullanım için önemli olabilir. LVS, travma veya işlemin kendisinin doğasında olabilen beyin hasarları için riskli hastalarda güvenle kullanılabilir.*

Anahtar kelimeler: *nörotoksisite, in vitro, levosimendan, NB2a, MTT*

Received/Geliş: 24.06.2020
Accepted/Kabul: 17.08.2020
Published Online/Online yayın: 21.09.2020

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INTRODUCTION

Levosimendan (LVS) and OR-1896, which are the long-lasting active metabolites of LSV, exert positive inotropy via a $Ca2^{+}$ sensitizing mechanism on the myocardium at their maximal free plasma concentrations [1]. In vitro experimental studies suggest that LSV, by opening the K (ATP) channels, has cardioprotective effect through anti-apoptotic and pro-autophagic properties [2]. The improvement of myocardial tissue perfusion (coronary blood flow), and its ability to decrease pre- and after-load, contributes to the cardioprotective effects of LVS [3]. LSV strengthens contractions of myocardium without increasing consumption of oxygen and it has anti-oxidant, anti-inflammatory, inotropic and vasodilatatory effects. With these properties, LSV is preferred in patients who have decreased ejection fractions during cardiac surgery.

Neuroprotective effects of LVS have also shown in a traumatic brain injury (TBI) model. Levosimendan is associated with modification of regional blood flow and cardiac output. This mechanism of action may also modulate functions of other organs as well.. Thus, LVS can protect neurons indirectly by increasing cardiac output or cerebral blood flow [4-8]. It has been shown in in-vitro traumatic brain injury and middle cerebral artery occlusion models that LVS reduces neuronal damage [4,9]. However, the direct effect of LVS on neurons is yet to be elucidated.

The mouse neuroblastoma cell line (NB2a) is a sensitive predictor of neurotoxicity, and its relative ease of culturing and reproducibility suggest that it is suitable for further use and development of experiments involving the assessment of interactions [10]. NB2a cell line is frequently used in the examination of neurotoxic effects on the neuronal cells. The neurotoxic effects of drugs are evaluated by percent inhibition of neurites. If the damage is moderate, the nerve cell withdraws the neurite outgrowth with or without apoptosis whereas high toxic effect ends up with necrosis [11].

The differentiation of neurons in culture, evidenced by neurite outgrowth is a general indicator of cellular well-being. Measurement, of the length of the neurite therefore, provides a useful in vitro model for the assessment of neurotoxicity and has been successfully used to demonstrate the neurotoxic potential of a wide range of agents, including excitatory amino acids. Neurite outgrowth is a specific structural end-point unique to the nervous system and depends upon a number of critical cellular processes, such as axonal transport. The inhibition of neurite outgrowth is only one marker of neurotoxicity that involves differentiating cells; thus, it may be more relevant to the exposure of the developing nervous system, rather than the mature nervous system [10,11].

Many studies have showed that LVS protects neurons indirectly. However, its direct effect on neurons is not fully understood yet. Therefore, we examined whether LVS has a direct neuroprotective effect using neuroblastoma cell line of mouse origin (NB2a).

MATERIAL and METHODS

Ethical Approval

Ethical Approval was given by the Medical Ethics Committee of Manisa Celal Bayar University (No: 10.05.2018-20.478.480).

Material

Mouse NB2a neuroblastoma cells were acquired from European Collection of Cell Cultures (ECACC, cell line: 89121404). All the chemicals used in the experiment were obtained from Sigma Pharmaceuticals St. Louis, MO, USA). Tissue culture flasks and culture plates were supplied by Falcon/ Fred Baker (Runcorn, Cheshire, UK) and gentamycin (GentaR 20 mg vial) was procured from Ibrahim Ethem Pharmaceutical Company, Istanbul, Turkey. LVS was procured from Sigma Pharmaceuticals, St. Louis, MO, USA) and diluted by dissolving in ethyl alcohol at 1/5 (0.2%) final concentration and the

cells were incubated for a further 24 hours.

Cell Culture

N2a cells proliferated in culture flasks with glucose-rich Dulbecco's Modified Eagle Medium (DMEM) with Glutamax-1, containing 5% fetal calf serum, 5% horse serum, 1% penicillin/streptomycin solutions (10000 U/10 mg) and 25 µg/mL gentamycin in incubator humidified with 5% CO_2 at 37°C.

Cell Viability

In the MTT assay, reduction of 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide to a purple formazan product, was used to estimate cell viability and growth.

LVS at concentrations of 0.1, 0.3, 1, 3, 10, 30 and 100 µM were used for measurement. Cell suspensions were first prepared at densities of 50.000 cells /mL per each well of 96-well culture dishes and plated in triplicate for each concentration. Growth Medium (100 µL) without LVS was used as a positive control. Cells treated with different concentrations were incubated for 24h. In the last 4h of the culture period, cells were incubated in a humidified incubator with 5% CO_2 at 37°C with MTT. The medium was then decanted and 200 µL dimethylsulfoxide (DMSO, Sigma-Aldrich) was added to each well to ensure dissolution of the formazan salts. The absorbance was immediately determined at 570 nm in an UV-visible spectrophotometer multiplate reader (Versa Max, Molecular Device, Sunnyvale, CA) and data were recorded [12].

Measurement of Neurite Outgrowth

In order to measure neurite outgrowth, NB2a cells were plated in the proliferation medium on to 24-well culture plates at a cell density of 15.000 cells/mL. Twenty-four hours later, the cells were induced to differentiate and generate neurites in the presence of the LVS with the following method: the culture medium within each well were discarded and replaced with serum-free medium plus 0.5 mM dibutyryl cyclic AMP containing LVS at concentrations of 0.1, 0.3, 1, 3, 10, 30 and 100 for measurement of neurite outgrowth. Cells were fixed with 4% (w/v) paraformaldehyde in phosphate buffered saline (PBS) for 10 min at a temperature of 24°C, then were stained for 3 min with Coomassie Blue cell dye (0.6% [w/v] Coomassie Brilliant Blue G in 10% [v/v] acetic acid, 10% [v/v] methanol, and 80% [v/v] PBS), washed with PBS. Three blinded observers took photographs of samples using the Olympus BX-40 (Olympus, Tokyo, Japan) light microscope equipped with a video camera (JVC-TK-C 601, Tokyo, Japan) for digital imaging. Image analyses were performed using Image-Pro Plus image analyser (5.1, Bioscience Technology, Bethesda, MD, USA). For drug and control groups, 10 different fields with approximately 10 cells were selected. A software routine was written using the functions of the image analyser to enable the automatic measurement of the total length of neurites (in pixels) in a given field and to express the results as the mean length of neurites per cell [10].

TUNEL Assay

Terminal deoxynucleotidyl transferase-biotin nick endlabelling (TUNEL) assay using the DeadEndTM Colorimetric TUNEL system (Promega, Madison, WI, USA) was employed to detect apoptotic cells. Following the procedure, cells were fixed in 4% paraformaldehyde for 30 min and rinsed three times in PBS for 5 min. Cells were then incubated with 20 µg/mL proteinase K for 10 min and washed three times again in PBS for 5 min. For inhibition of endogenous activity, cells were treated with 3% hydrogen peroxide and rinsed in PBS. Cells which were treated with equilibration buffer for 5 min were incubated with Tdt-enzyme for 60 min at 37°C, processed with 2×SCC solution for 15 min and then washed three times in PBS for 5 min. Streptavidin peroxidase procedure was performed for 45 min, after which cells were rinsed in PBS and incubated with DAB; Mayer's hematoxylin was performed for counterstaining. Cells were then rinsed in distilled water and mounted in the mounting medium. TUNEL-positive staining was evaluated by the blinded observer using an Olympus BX40 light microscope [10].

Apoptotic Index

Apoptotic index was defined as the ratio of positively labelled cells to all cells in selected fields. For TUNEL staining, each section was counted for 100 TUNEL-positive cells in randomly chosen fields. The percentage of apoptotic cells were also checked out by a blinded observer and scored as 0: no apoptosis, 1: 1%-10% apoptosis, 2:11%-25% apoptosis, 3: 26%-50% apoptosis, 4: 51%-75% apoptosis, and ≥ 5: 75% apoptosis [13].

Statistical Analysis

The results were analysed using GraphPad (SanDiego, CA, USA) using one-way ANOVA with Tukey post- hoc testing and presented as mean±SEM. Statistical significance was defined as $p<0.05$.

RESULTS

In the cell proliferation phase, LVS was applied at 0.1, 0.3, 1, 3, 10, 30 and 100 µM concentrations, and neurotoxic effects were analyzed by MTT assay. Cell proliferation decreased above 10 µM concentrations of LVS compared to the controls, but it was not statistically significant (Figure 1). LVS was left for 24 h after the differentiation for neurite inhibition (Figure 2). LVS neither showed any neurotoxic effect nor caused neurite inhibition ($p>0.05$).

NB2a cells were taken into the culture medium and left to proliferate. Then, we waited for further 24h for the neurite outgrowth in the differentiation medium, and the cells were stained with Coomassie Blue. Differentiation of NB2a cells to neurons by adding d-cAMP to serum- free medium at 48h is presented in Figure 1a. The neurites of cells in proliferation medium didn't outgrow and cells looked healthy and in good shape morphologically. The image of cells of negative control and NB2a cells with 1 µM LVS are presented in Figure 3.

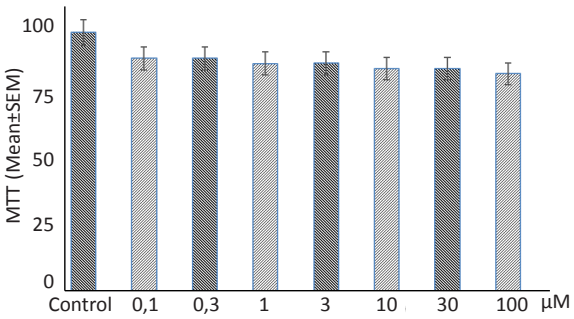

Figure 1. The effect of different concentrations of LVS on cell viability and proliferation by using MTT (methylthiazol diphenyltetrazolium) assay. Results are expressed as percentage of controls (mean ±SEM) over 3 experiments of (P>0.05).

The toxic effect of LVS on apoptosis in NB2a cells was analysed by TUNEL assay. There were no significant differences in the apoptotic cell index of LVS at concentrations of 10 and 30 µM compared to the control groups (Figure 4).

The effect of LVS at different concentrations on the neurite outgrowth was measured by image analysis. Mean (±SD) of percentage results were compared with those of controls. There were no significant differences between two groups (Figure 5).

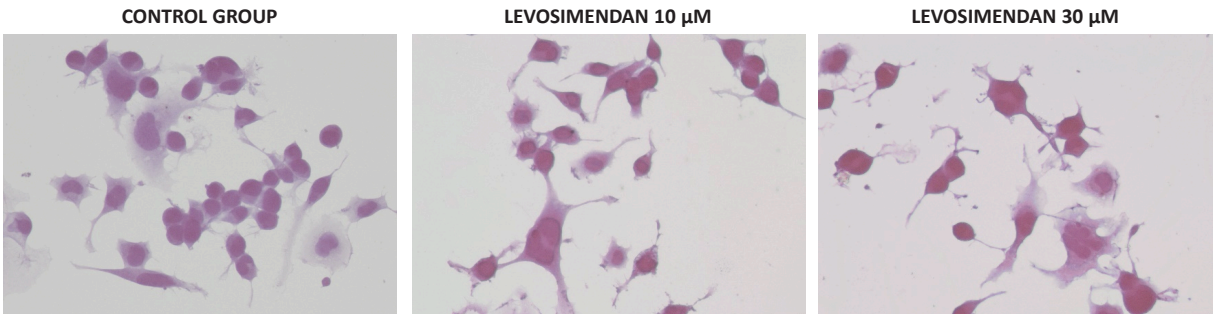

Figure 2. NB2a cells were observed with quite healthy morphology and neurite outgrowths at 10 and 30 µM concentrations of LVS. There were no signs of apoptosis by TUNEL.

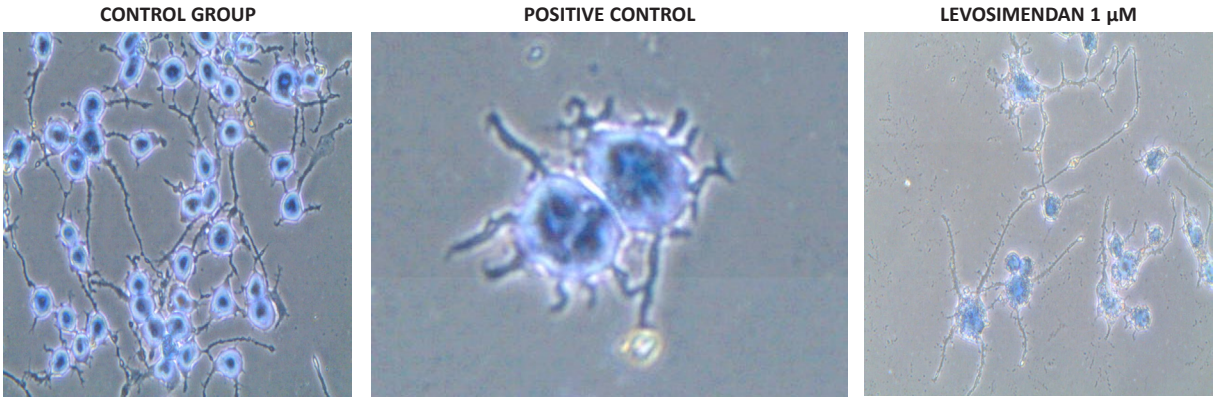

Figure 3. Differentiation NB2a cell to neurons by adding d-cAMP to serum free medium (Upper). The image of the negative control cells (Middle). The neurite extension induced by 1 µM LVS on NB2a cells (Lower) (Coomassie Blue stain, X400).

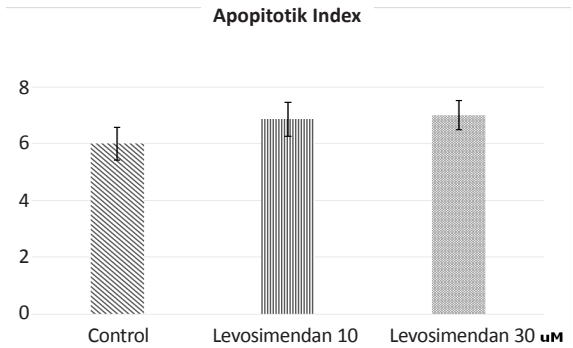

Figure 4. Following treatment of NB2a cells with LVS at 10 and 30 µM concentrations, there were no significant increases in the number of apoptotic cells of LVS compared to the control group (p>0.05).

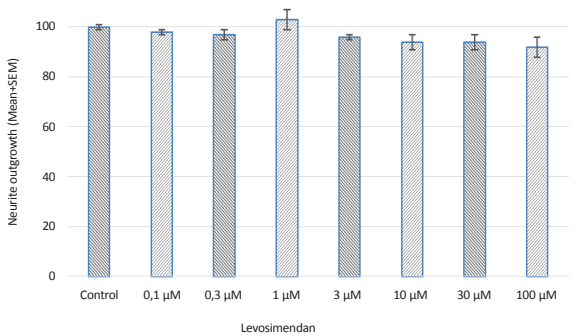

Figure 5. The effects of different concentrations of LVS (µM) on the neurite outgrowth. Results are expressed as mean ± SEM of percentage compared to the controls (p>0.05).

DISCUSSION

We explored the neuroprotective effect of different concentrations of LVS on the mouse neuroblastoma NB2a cell line. We found that LVS did not have any harmful effect on neurite inhibition cell viability and apoptosis. We also found that LVS did not inhibit neurite extension in neuroblastoma NB2a cells at any concentration. Furthermore, LVS at 1 µM concentration increased the neurite outgrowth significantly, compared to the control groups. Although neuroprotective effects of LVS have been reported in various experimental brain injury models, this study, to the best of our knowledge is the first one which explored the direct neuroprotective effect of LVS on neuroblastoma NB2a cell line.

Clinically recommended therapeutic concentrations of LVS have been reported as 0.03 µM to 0.3 µM for Ca+2 sensitizing effect and greater than 0.3 µM for PDE-III inhibitor effect. Therefore, we used these concentrations in culture conditions. We preferred higher than therapeutic concentrations to identify its toxic effects. Our study showed that these concentrations of LVS did not have harmful effects on neurons. Moreover, LVS at 1 µM concentration increased the length of neurites demonstrating its effect beyond neuroprotection.

Neurite outgrowth is a basic neuronal function that depends on axonal transport, microtubule recombination and neurofilament proteins. Biological and toxic environmental substances can inhibit neurite

outgrowth; therefore, monitoring neurite growth can be used to investigate the neurotoxic activity. Mechanisms which play a crucial role in neurite outgrowth in response to cyclic AMP have been investigated. It has been shown that nitrous oxide-cGMP-PKG pathway contributed to neuritogenesis is one of these in vitro mechanisms Therefore, another way for LVS to induce neurite outgrowth is through an NO-cGMP-PKG signaling pathway followed by ERK activation with the involvement of TrkA in Neuro2a cells [14]. Based on our results, it is possible that LVS increases neurite outgrowth at 1 µM via these signal mechanisms.

Many experimental animal models in literature, namely in vitro model of traumatic brain injury, spinal cord ischemia/reperfusion injury models, cortical neuron ischemia/reperfusion injury models and asphyctic cardiac arrest in rats, have shown that LVS reduces neuronal damage and has neuroprotective effect (4, 6, 8, 9, respectively). Despite its anti-inflammatory and peripheral anti-apoptotic effects, levosimendan did not show a long-term neuroprotective effect in septic encephalopathy models in rats [15].

Neurological complications can be observed after cardiac surgery, causing significant morbidity and mortality. Guerrero-Orriach and co-workers reported an 83% incidence of delirium and cognitive change after cardiac surgery [7]. LVS may be protective against these complications due to its neuroprotective effects [5]. It is also possible that the positive effects of LVS on neurological function may be a result of improved organ perfusion, cardioprotection and inodilation. Thus, LVS-induced vasodilatation and subsequent increase in the blood flow to the central nervous system may partially be related to lowering of intracellular free calcium through potential inhibition of phosphodiesterase III, calcium desensitization, or opening of adenosine triphosphate-sensitive potassium channels [1,2,4-6].

Another possible explanation of its direct neuroprotective effect is its antiapoptotic property. LVS acutely opens the mitoKATP channels, resulting in K^+ influx, organelle depolarisation, and the expansion of mitochondrial matrix volume. Mitochondrial Ca^{++} overload has been closely correlated with mitochondrial damage, which can result in both necrotic and apoptotic forms of cell death [9]. Despite the profound differences in cellular physiology and sensitivity to anoxic injury that exist between myocardial cells and neurons, LVS has been shown to protect the heart and as a result of its mechanism of action [16,17], Iit is possible that it also protects the brain as well. Shimizu et al have shown that diazoxide, a selective Mito KATP canal opener has a neuroprotective effect against ischemia/reperfusion injury in rat cortical neuron and these effects are reversed by selective Mito KATP canal antagonist 5-hydroxydecanoate [8]. In our study, compared to the control group LVS at concentrations of 10 and 30 µM did not trigger apoptotic pathway. In contrast to our study, LVS failed to induce a neuroprotective effect in rat sepsis model in which the authors investigated the long-term effect of LVS on brain in comparison to its peripheral mode of action [14]. Moreover, Roehl et al, found that although LVS has neuroprotective activity in vitro and in vivo protective activity on the spinal cord, LVS also crosses the blood–brain barrier. LVS did not alleviate the initial neuronal injury after transient ischaemia/hypoxia model [5].

Neuroblastoma cells from Na2 cell line need two factors in order to differentiate to neurons. First one is dibutyl-cAMP which causes cells to outgrow neurite for neuronal differentiation. The other is removal of serum from the medium and thereby changing the medium from a proliferation to differentiation medium with minimum serum concentration. Differentiation medium makes these cells to become neurons by stimulating neurite extensions. Typical mature and differentiated neurons with long neurites develop under the effect of these two factors [18]. The mechanisms balancing proliferation and differentiation of neuroblastoma cells are poorly

understood. Neuroblastoma cells exhibit the fascinating potential to be able to differentiate spontaneously and regress. Signaling pathways that would promote differentiation of neuroblastoma cells are intriguing candidates for experimental approaches.

However several agents have been identified that induce neuronal differentiation of neuroblastoma cells in vitro, including retinoic acid and growth factors such as neurotrophins [19]. Previous work exploring downstream pathways established that neurotrophins signal via receptor tyrosine kinases (RTKs), which in turn activate the Ras/Raf/MEK/ Erk1/2 and PI3K/Akt signaling modules, leading to execution of the complex transcriptional programs necessary for growth arrest and morphological differentiation [20]. Evangelopoulos et al. reported that the activation of the EGFR by mevastatin triggers neurite outgrowth of neuroblastoma cells. Mevastatin and serum withdrawal triggered the production of NO. In addition, the differentiation of Neuro2a cells and the activation of Akt/PKB triggered by serum withdrawal could be blocked by addition of the NO synthetase (NOS) inhibitor l-NAME. Moreover, mevastatin and serum withdrawal rapidly increased the expression of the neuronal NOS isoform nNOS. However, addition of single nucleotide polymorphism (SNP) as an NO donor per se did not trigger neurite outgrowth. They reported the role of NO in neurite outgrowth of neuroblastoma cells triggered by mevastatin or serum reduction [20].

CONCLUSION

We found that there was no sign of harmful effect of LVS on viability and proliferation. We did not observe any sign of cell death with LSV in any concentrations on neuroblastoma NB2a cells. Moreover, LVS at 1 µM concentration significantly increased the neurite outgrowth. LVS can safely be used in patients who have a risk for a brain injury due to the nature of disease, trauma or the procedure of cardiac Further studies are needed to reveal the mechanisms responsible of neurite outgrowth.

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