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Research article

Valproic acid preserves motoneurons following contusion in organotypic spinal cord slice culture

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Objective: Spinal cord injury (SCI) is a devastating condition causing neuronal loss. A key challenge in treatment of SCI is how to retain neurons after injury. Valproic acid (VPA) is a drug recently has been appreciated for its neuroprotective and neurotrophic properties in various SCI models. In this study the role of VPA was assessed in organotypic spinal cord slice culture following the contusion.

Design: The lumbar enlargement of adult rat was cut transversely and slices were cultured. Seven days after culturing, injury was induced by dropping a 0.5 gram weight from 3 cm height on the slice surface. One hour after injury, the VPA was administered at 1, 5 and 10 μ M concentrations. Afterward, at day 1 and 3 post injury (DPI: 1 and 3) propidium iodide (PI) and immunohistochemistry staining were performed to evaluate the cell death, NeuN and β -Tubulin expression, respectively.

Results: The PI staining of slices at DPI: 1 and 3 following treatment with VPA revealed significant decreases in the cell death in all three concentrations comparing to the non-treated group. Also immunostaining showed VPA only at 5 μ M concentration considerably rescued ventral horn' MNs from death and protected the neuronal integrity.

Conclusion: The results of this study indicate applying VPA one hour after injury can prevent the death of a majority of cells, importantly MNs and preserve the neuronal integrity. Since the first 24 hours after SCI is a critical period for employing any treatment, VPA can be considered as an option for further evaluation.

Keywords: Contusion, Spinal cord, Traumatic injury, Valproic acid

Introduction

Spinal cord injury (SCI) is a devastating condition causes to irreversible neuronal loss, which ultimately leads to persistent neurological dysfunction. Currently, various treatment options have been employed for SCI, including the use of high-dose methylprednisolone, surgical intervention to decompress and stabilize the spinal cord and rehabilitative care. Also, diverse promising neuroprotective, cell-based strategies and neuroregenerative materials are being tested for SCI.¹

Due to the complexity of SCI, presently there is no fully restorative treatment and there is a consensus among researchers that combination therapies will be more effective compared to the single therapeutic

strategy alone.² Since the majority of approaches used to cure are time consuming, employing a fast pharmacological treatment during the therapeutically optimal time window can be a potential strategy after SCI. The use of pharmacological substances before any other treatment not only can diminish the inhospitable milieu of injury but also can provide a better environment for subsequent therapies which may work synergistically.³

Valproic acid (VPA, 2-propylpentanoic acid), a short-chain fatty acid, widely used in clinical practice for treatment of seizures and bipolar mood disorders for more than four decades recently has been appreciated for its neuroprotective properties.⁴⁻⁶

It has been reported that VPA at therapeutic doses exerts its neuroprotective impact during the secondary phase of injury by attenuating SCI-induced apoptosis,

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neurotoxicity, inflammation and autophagy.^{7,8} Although all these benefits of VPA have been investigated in the animal models of SCI, still little is known about the function of this drug in *ex vivo* models.

To the best of our knowledge, there are two reports that used an *ex vivo* model of SCI to evaluate VPA impact; both studies reported that injury was induced by the chemical agents that only mimic the secondary phase of injury.^{9,10} Although the mechanically-induced model of injury can simulate both primary and secondary phase of injury, our knowledge about the effects of VPA in this context is limited. Therefore this study was designed to assess the role of VPA in recently developed organotypic spinal cord slice culture obtained from adult rat following the contusion.

Methods

In the present study, all animal experiments were undertaken in accordance with the Ethical Committee for the use and care of laboratory animals of Neuroscience Research Center, Shahid Beheshti University of Medical Sciences. At any experimental analysis, nine slices were used which obtained from three different rats.

Spinal cord organotypic slice culture

The organotypic spinal cord slice culture was prepared according to the standard interface method.¹¹ The adult rat was deeply anesthetized with CO₂ and back skin was removed and the vertebral column was dissected. Once exposed, the spinal cord was flushed out with ice-cold PBS using a 60-ml syringe and placed into a petri dish containing sterile, ice-cold HBSS (Hank's Balanced Salt Solution, Sigma-Aldrich Co, LLC, St. Louis, MO, USA). Then, the lumbar enlargement region of cord was excised and embedded in the low melting point (LMP) agarose (4% V/W in HBSS, Promega Corp, Madison, WI, USA). Subsequently, the embedded spinal cord was cut transversely at 400 μ m thickness. The sections were checked under stereomicroscope (Carl Zeiss Meditec Group, Dublin, CA, USA) and undamaged slices were transferred on the Millicell culture plate inserts (PTFE membrane, 0.4 μ m pore size; MilliporeSigma, Billerica, MA, USA). The culture inserts were placed into a six-well plate (PTT, Switzerland) with 1 ml of culture medium consisted of 50% minimum essential medium (MEM) with L-glutamine (Sigma-Aldrich Co, LLC, USA), 25% heat-inactivated horse serum, 25% HBSS (Sigma-Aldrich Co, LLC, USA), 6.4 mg/ml D-glucose (Sigma-Aldrich Co, LLC, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) (Fig. 1).

Protocol for spinal cord contusion

Following 6 days *in vitro* condition SCI was induced at day 7 (DIV: 7) using a weight drop device adopted from Krassioukov *et al.*¹² Briefly, an impactor with a 3 mm diameter head and 0.5 gram total weight was dropped from a height of 3 cm on the entire slice surface and subsequently slices were returned to incubator (Fig. 1).

VPA administration

Valproic acid was provided by the Darou Pakhsh Pharma. Chem. Co. (Tehran, Iran) and 1, 5 and 10 μ M concentrations of this drug were prepared in the complete medium (1% V/V) and have been applied one hour following injury.

PI staining

The identification and quantification of dead cells in the cultured slices of spinal cord were carried out using the Propidium Iodide (PI, Invitrogen, USA) as a fluorescent indicator for the loss of plasma membrane integrity. At first and third days post injury (DPI:1,3), both injured and VPA treated slices and their uninjured counterparts were separately introduced to 1 ml PI solution at 5 μ g/ml concentration, prepared in serum free medium. Then slices placed back into the incubator for 30 minutes to allow permeation of PI. Here four different regions of the grey matter in each slice were analyzed (Fig. 2A).

The average number of the PI⁺ cells counted in the four quadrants of each slice was considered as a single mean which has been normalized by dividing to average number PI⁺ cells in the non-injured group.

Immunohistochemistry

The spinal cord slices of both injured and VPA treated groups at the day one and three following injury were fixed for 2 h in 4% paraformaldehyde at RT, followed by overnight cryoprotection in the 30% sucrose (Merck, Munich, Germany) at 4°C. Subsequently, slices were embedded in OCT (optimum cutting temperature, Sakura Finetek Japan, Tokyo, Japan) and sectioned at 12 micrometer with cryostat (Histo Line Laboratories S.r.l., Milan, Italy) and stored at -80°C.

Immunostaining was carried out on the 12 μ m thick cryosections. Sections were fixed with acetone (Merck, Germany) for 20 min, then quenched in the 1% hydrogen peroxide (Merck, Germany) and permeabilized in the 0.2% Triton X-100 (Merck, Germany). Here the 10% goat serum was used for blocking (Sigma, USA) and primary antibody was employed after dilution in blocking buffer and incubated overnight at 4°C. Next day, the sections were incubated with HRP-conjugated

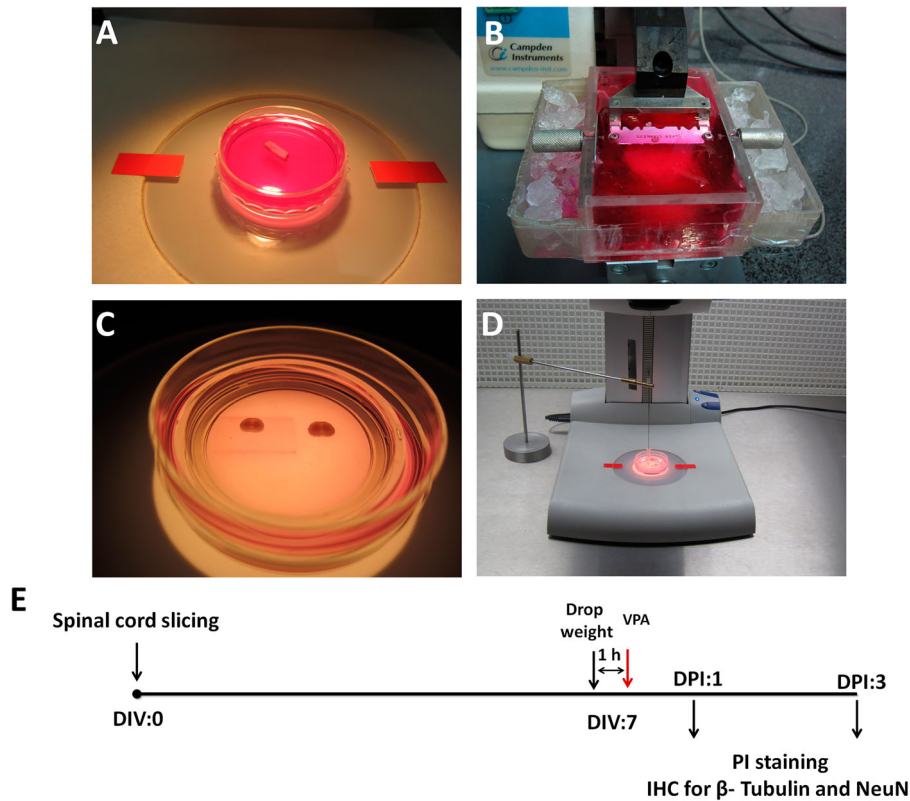


Figure 1 Preparation of SCI model in organotypic spinal cord slice culture and VPA treatment protocol. The lumbar enlargement of adult rat spinal cord was excised and embedded in 4% LMP agarose (A), then cut transversely with vibratome (B) and undamaged slices were transferred to millipore culture insert (D). After seven days *in vitro* injury was induced by dropping a weight on top of slices (E). The entire protocol of experiment was illustrated in Schematic sketch (F). LMP: low melting point agarose (Colour online).

secondary antibody (EnVision™+ Dual Link System-HRP, Dako Denmark A/S, Glostrup, Denmark) for 30 minutes at RT and subsequently the

diaminobenzidine chromogen solution (DAB, Dako, Denmark) was applied enough to cover the specimen to detect bound antibody. Finally they were immersed

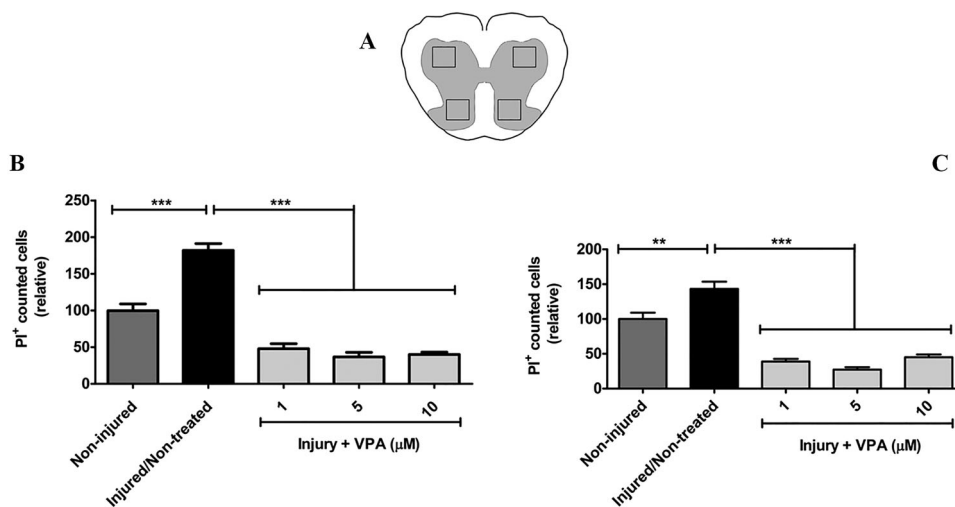


Figure 2 Evaluation of cell death by PI staining in VPA treated slices of spinal cord after injury. The number of PI⁺ cells was counted in the four regions of grey matter (A) Here the average number of PI⁺ cells counted in four quadrants of each slice was considered as a single mean which has been normalized by dividing to average number of PI⁺ cells in the non-injured group. The statistical analysis was performed on the first (B) and third day (C) after injury to compare the number of PI⁺ cells in the four mentioned regions of VPA treated injured slices with injured/non-treated and non-injured counterparts.

in a bath of aqueous hematoxylin for counterstaining. Primary antibodies were: mouse anti-NeuN (1:50, Merck Millipore, Germany) and rabbit anti- β III Tubulin (1:2000, Abcam, Cambridge, MA, USA).

Imaging and statistical analysis

The images were obtained by the Olympus invert fluorescence microscope (IX71) and the Nikon light microscope (E600). Statistical analyses were performed on GraphPad Prism (Version 5, 1992–2013 GraphPad Software, Inc., La Jolla, CA, USA) using one-way ANOVA. $P < 0.05$ was considered significant.

Results

Given various SCI models that currently exist to recreate features of human SCI, in this study the *in vitro* model of SCI was treated with various concentrations of VPA one hour following the injury. Here the average number of PI⁺ cells in the four regions of grey matter in each slice (defined square zones of sketch, Fig. 2A) was evaluated between the injured, VPA treated and non-injured counterpart groups at DPI: 1 and 3. The PI staining of slices on day first and third after drop weight injury (DPI: 1 and 3, non-treated injured group) showed a significant increased rate of cell death compared to the control group (non-injured counterpart). However, in slices treated with all three concentration of VPA (1, 5 and 10 μ M) one hour after injury the number of PI⁺ cells significantly decreased at both DPI: 1 and 3 compared to the non-treated injured counterpart (Figs. 2B, 2C).

To clarify the difference of neural integrity between slices of each group, immunostaining against β III Tubulin was performed on the 6 μ m frozen sections prepared from slices of non-treated injured and VPA treated groups. This staining demonstrated that treatment of injured slices with 5 μ M VPA considerably protected the neural integrity as the expression level of this neuron specific protein was higher in the treated slices than non-treated injured counterparts (Fig. 3).

In addition, to compare the number of ventral horn MNs between the injured and VPA treated groups, immunostaining was conducted against NeuN on frozen sections of each group slices (Fig. 4). Here quantitative analysis was performed on the total number of MNs counted in the three areas of each half of gray matter (Three defined square, Fig. 5A). The statistical evidences revealed that VPA at 5 μ M concentration significantly rescued MNs loss following injury on both first and third days post injury (Figs. 5B, 5C).

Discussion

Based on growing number of evidence, organotypic spinal cord slice culture has been considered as a promising replacement of animal models and it is a proper context for addressing the impact of numerous treatments following SCI.¹³

Pharmacological intervention is one of the potential strategies that have gained considerable insight to promote recovery after SCI alone or in combination with other treatments as an enhancer to attenuate the inhospitable environment of SCI for elevating the impacts of other subsequent main therapeutic strategies like cell-based therapies.^{14–16} The delivery of a pharmacological agents with neuroprotective role shortly after SCI has been long explored. Up to the present, a variety of enhancing drugs have been tested in the animal SCI models. Since modeling injury in organotypic spinal cord slice culture is rapidly increasing,^{13,17} finding the effective therapeutic interventions in this new context is required for future combinatory therapies.

The present study provides evidence of VPA neuroprotective effects in an *in vitro* model of spinal cord injury. Here it was revealed that VPA treatment of the mechanically injured spinal cord slices at 5 μ M concentration one hour following the injury can preserve the neural integrity and prevent cell death specifically ventral horn MNs mortality.

To the best of our knowledge, this is the first investigation that aimed to evaluate the VPA impact on the mechanical induced model of injury simulated in the organotypic spinal cord slice culture. Currently growing body of evidence reports VPA as a main therapeutic approach that overcomes biological complications caused by injuries through its neuroprotective effects in animals^{18–20} and chemically induced organotypic SCI models. However, recently substantial efforts have been devoted to elucidate the enhancing role of VPA when it employed with various stem cell therapies *in vivo*.^{21,22} Here in parallel, in order to evaluate the future combinatory impact of VPA with various cell based therapies in the *ex vivo* model of injury, we first assessed the beneficial effects of VPA in the organotypic context.

In this regard, three different concentrations of VPA were administered to the slices one hour after injury, which has been reported as a critical period, and subsequently the impact of VPA was evaluated on the first and third day after injury. In consistent with the study of Sugai and his colleagues, all three selected concentrations of VPA including 1, 5 and 10 μ M significantly

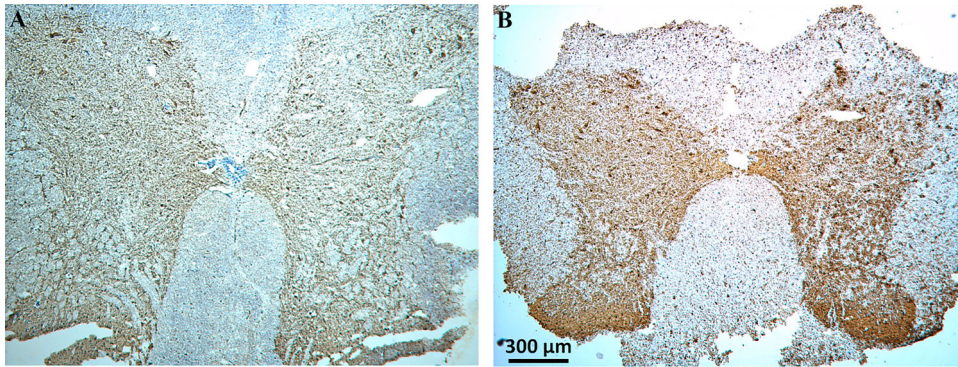


Figure 3 Expression of β III Tubulin in the spinal cord slices treated with VPA following injury. The immunostaining against β III was performed on the 6 micrometer sections obtained from non-treated injured slices (A) and 5 μ M VPA treated ones (B) on the first day after injury to compare the expression level of this protein (Colour online).

reduced the rate of cell death on both days comparing to the non-treated injured counterparts. Based on emerging evidences, a large population of neurons and glia dies right after injury and it has been repeatedly reported that SCI induced cell death is mainly associated with the endoplasmic reticulum (ER) stress and oxidative stress caused by injury.^{23,24} Valproic acid as a well-known histone acetylase inhibitor (HDAC) exerts its neuroprotective impact by reducing the progressive accumulation of the proapoptotic factor C/EBP homologous protein in the nucleus and caspase-12 to prevent the ER stress. In addition,

VPA administration was found to protect neuronal cells from the oxidative stress by inhibiting the release of cytochrome C, the impediment of caspase-9 and suppressing of reactive oxygen species, the superoxide anion and the inducible nitric oxide synthase.^{9,25–28} Although a reduced rate of cell death was also observed in our study, the beyond neuroprotective role of VPA is not clear here. Since most aforementioned protective mechanisms have been investigated in the animal models of injury, further examination is required to define the exact mechanism of VPA in this injured *ex vivo* platform.

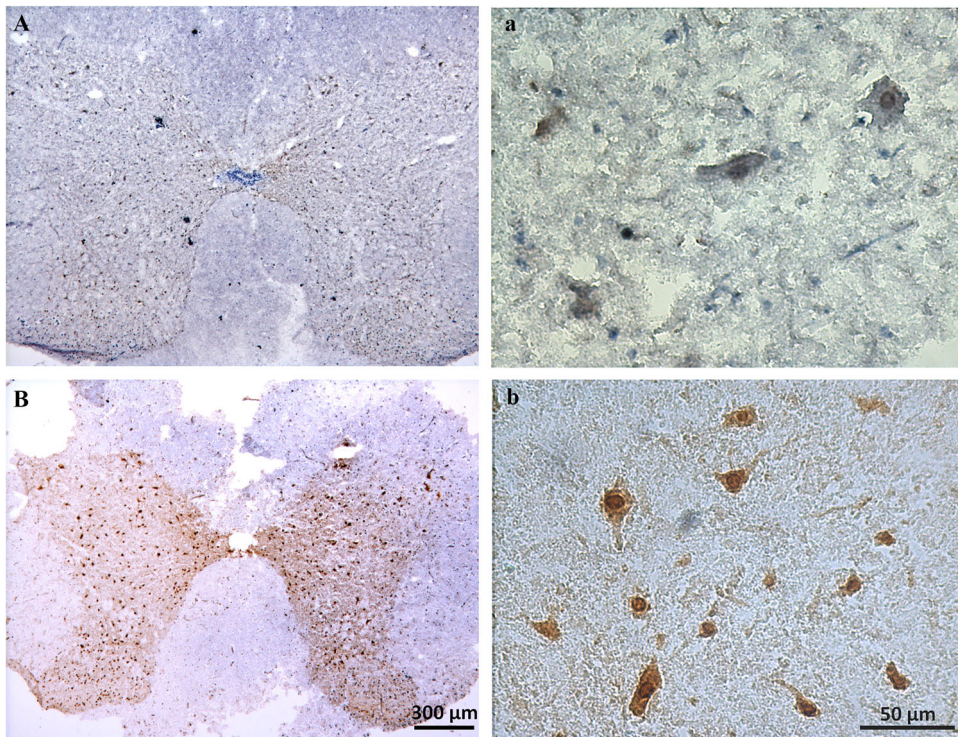


Figure 4 Evaluation of VPA impact on the ventral horn MNs following the injury. The representative micrographs stained by NeuN revealed the impact of injury (A, a) and VPA treatment (B, b) on the number of spinal cord ventral horn MNs at two magnifications (Colour online).

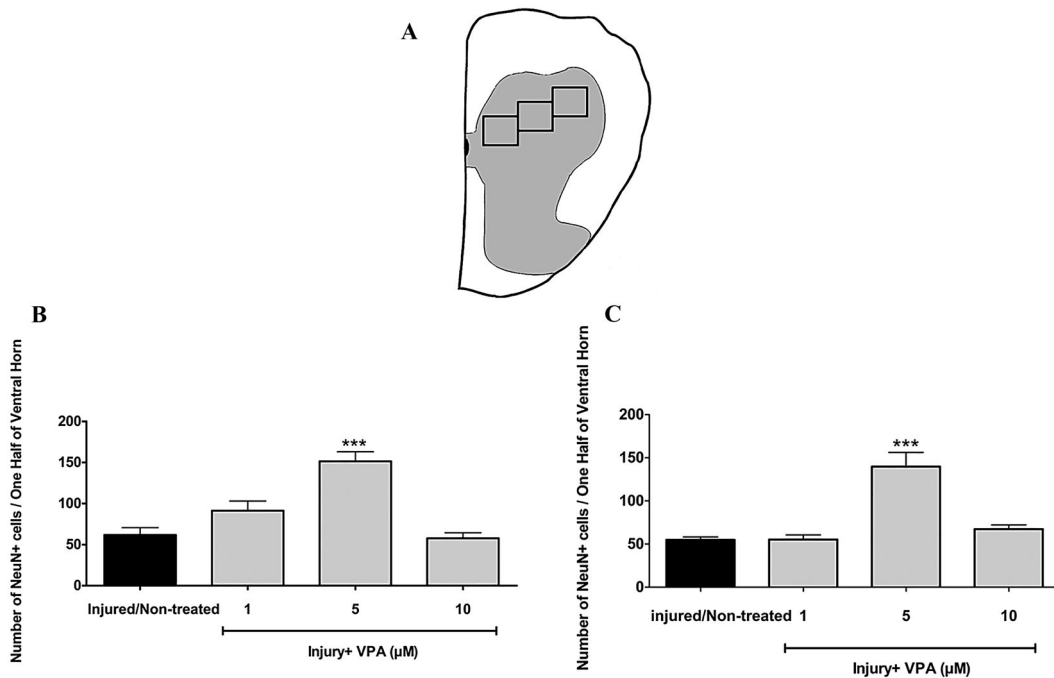


Figure 5 The quantitative analysis of VPA treatment on ventral horn MNs after spinal cord injury. The quantitative analysis of total number of NeuN⁺ cells at three different regions of each half of spinal cord (A) was performed on the first (B) and third day (C) post injury to compare the MNs survival between the non-treated injured and VPA treated groups.

However, all three therapeutic concentrations of valproic acid alleviated the rate of cell death, only 5 μM VPA can significantly reduce ventral horn MNs loss presumably through above aforementioned mechanisms. Here the 5 μM VPA was the optimized concentration that not only protects all spinal cord cells but also preserves neural cells, more specifically MNs. Additionally, finding of our study revealed expression of βIII Tubulin as an indicator of integrity can noticeably be preserved by VPA at 5 μM concentration. Since βIII Tubulin is a prominent protein mainly expressed by neural cells, its expression decreases after injury due to significant loss of neural cells.

Conclusion

Given the fact that majority of approaches used to cure SCI are time consuming, employing a fast pharmacological treatment during the therapeutically optimal time window can be a potential strategy. The use of a pharmacological drug like VPA before any other treatment not only can attenuate inhospitable context of injury but also provide a suitable environment for subsequent therapies which may work synergistically.

In this study the role of VPA was assessed in the newly developed organotypic spinal cord slice culture prepared from adult rat following the contusion. The results of present experiment indicate applying VPA one hour after injury can diminish death of majority of cells and

preserve neuronal integrity. Since first 24 hours after SCI is a critical period for employing any treatment, VPA can be considered as an option for further evaluation. Although most studies reported that VPA impose its neuroprotective role through its inhibiting action on histone deacetylation,^{4,5} it cannot be strongly ruled out that VPA exerts all its effects under the epigenetic control and further experiments are required to uncover its whole mechanism of action after SCI in the organotypic context.

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Disclaimer Statements

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Conflicts of interest The authors declare that they have no conflict of interests.

Ethics approval None.

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