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The effect of rifampicin on the neuronal cell survival in primary cortical neuron culture after laser axotomy

Lazer aksotomi sonrası primer kortikal nöron kültüründe rifampisinin nöronal hücre sağkalımı üzerine etkisi

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ABSTRACT

Aim: Neurodegeneration caused by the axonal injury is a widely seen phenomenon in spinal cord and traumatic brain injuries. Due to the disintegration of the synaptic connection neurotrophic factors could not be transported retrogradely towards the cell body and the deprivation of the trophic factors lead to the degeneration and death of the injured neuron. Rifampicin is an antibiotic exhibiting several neuroprotective functions in various neurodegenerative conditions. Here we aim to investigate the acute neuroprotective effect of rifampicin in primary cortical neuron culture in which neurons are axotomized by laser axotomy.

Methods: Neonatal male mice were used in order to isolate cortical neurons. Isolated primary cortical neurons were cultured. After 24 hours three different rifampicin concentrations (1 µM, 10 µM and 100 µM) were applied to the neurons and after 15 minutes of rifampicin addition, neurons were laser axotomized. Viability of the cells was evaluated by propidium iodide staining after 24 hours of axotomy.

Results: Laser axotomy decreases the cortical neuron viability significantly by 80.45%, while rifampicin pre-treatment increases their viability in all three dosages in a statistically significant manner.

Conclusion: Rifampicin has an acute neuroprotective effect on the viability of the laser axotomized cortical neurons.

Keywords: Laser axotomy, rifampicin, neuronal viability, primary cortical neuron, neurotrauma

ÖZ

Amaç: Aksonal hasarlanma sonucu oluşan nörodejenerasyon travmatik beyin hasarı ve omurilik yaralanmalarında sıklıkla görülen bir tablodur. Sinaptik disintegrasyon nedeniyle trofik faktörlerin nöron gövdesine retrograd olarak taşınamaması sonucu trofik faktör yetersizliği aksonu hasarlanmış olan nöronun ölümüne yol açar. Rifampisin antibiyotik etkilerinin yanı sıra çeşitli nörodejeneratif süreçlerde nöroprotektif etkileri de olan bir antibiyotiktir. Bu çalışmadaki amacımız lazer aksotomi ile hasarlanmış primer kortikal nöronlarda rifampisinin akut nöroprotektif etkilerinin araştırılmasıdır.

Yöntemler: Yenidoğan farelerin kortikal nöronları izole edilerek kültür edildi. Yirmi dört saat sonra seçilen üç rifampisin dozu (1 µM, 10 µM ve 100 µM) primer kortikal nöronlara uygulandı. Rifampisin uygulamasından 15 dakika sonra lazer aksotomi gerçekleştirildi. Aksotomiden 24 saat sonra propidiyum iyodit boyaması ile nöronların canlılığı ölçüldü.

Bulgular: Lazer aksotomi nöronal canlılığı %80,45 oranında azalmaktadır. Buna karşılık rifampisin ön uygulaması ise aksonları hasarlı nöronların canlılığını seçilen üç dozda da anlamlı ölçüde arttırmıştır.

Sonuç: Lazer aksotomi ile hasarlanmış kortikal nöronlardaki canlılığı arttırması nedeniyle rifampisinin söz konusu deneysel modelde akut nöroprotektif etkisi olduğu düşünülmektedir.

Anahtar Kelimeler: Lazer aksotomi, rifampisin, nöronal canlılık, primer kortikal nöron, nörotravma

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INTRODUCTION

Axotomy usually triggers the cellular changes and alterations in gene expression profiles in neurons. It also affects the transport of several neurotrophic factors through axons and leads to the degeneration of neurons in a retrograde fashion. This degenerative process is caused widely by the deprivation of target-derived trophic support; the nature of the abnormalities seen in the axonal injury depends on the type of axotomized neuron, the intensity of the injury and the site of axonal injury, especially the distance of the injury site to the cell body [1].

Several studies reveal that significant differences have been observed after axotomy in certain neurons indicating that a variability exists in the applied axonal trauma's severity [1]. In order to control the variability in the axonal trauma models, in vitro axonal injury methods have been developed in controlled cell culture conditions. Although mechanical impact application and stretchers are among the most frequently preferred methods in these in vitro models, the accumulating evidence about these models show that they could not mimic the direct mechanical damage-related neuron injury, because it is very difficult to assess whether the applied mechanical insult by itself is the direct cause of the neuronal damage or the cellular damage is a secondary event [2,3,4]. Therefore, automatically-controlled microdissection of the cultured cortical neurons might be an alternative and appropriate in vitro neuronal trauma model to investigate the mechanical damage of the neurons with low-variability and high accuracy [3,5,6].

These high accuracy in vitro trauma models could provide a beneficial approach when combined with candidate neuroprotective agents. The relevant scientific literature about the in vitro application indicate that laser axotomy is a feasible neuronal trauma model to investigate the neuroprotective effects of canditate molecules in a more standardized experimental model [2-8]. This method not only provides a precise control on laser parameters but it also gives an opportunity to create total or localized axonal damage.

Rifampicin is a broad spectrum antibiotic, and in addition to its conventional anti-microbial effects, it also has neuroprotective effects in various neurodegenerative disorders, including Parkinson's Disease (PD), Multisystem Atrophy and Alzheimer's Disease (AD) [9-12]. In PD rifampicin increases dopaminergic neuron survival, decreases alpha-synuclein toxicity and by modulating the activities of chaperons and anti-apoptotic proteins it also decreases apoptosis [10,11].

It has also been shown that rifampicin modulates neuroinflammatory process and amyloid beta metabolism in AD [12]. It is well-known that one of the neuropathological hallmarks of AD is the accelerated accumulation of amyloid-beta plaques, and these plaques initiate the toxic processes in the brain [13-17]. Rifampicin inhibits amyloid-beta oligomerization and the resulting amyloid-beta monomers are less toxic than their fibril forms [14,15]. It has also been demonstrated that rifampicin is 10-100 fold more effective than vitamins in the inhibition of amyloid-beta accumulation [16,17,18]. Having its anti-oxidative and anti-inflammatory effects together with its capability for crossing blood-brain barrier, rifampicin is a good candidate for neuroprotective studies in different neurodegenerative settings.

Here we aim to investigate the possible role of rifampicin on the axonal damage which is generated by laser axotomy in an in vitro primary cortical neuron culture.

MATERIALS and METHODS

In this study 20 neonatal (P0-P1) Balb-C male mice were used and the study has been approved by the Ethical Committee of Experimental Animals of Istanbul Medipol University (38828770-604.0101-E.35856).

Primary Cortical Neuron Culturing

Neonatal mice were decapitated under 4% isofluorane anesthesia, cortical layer was taken carefully and meninges were removed. The dissection procedure was performed in ice-cold Leibovitz's Medium (L15) (Sigma-Aldrich, L5520, Germany) containing 2 mM glutamax (Gibco, 35050061, USA), 100 U/100 µg penicillin/streptomycin, 250 ng amphotericine (Sigma-Aldrich, A5955, Germany), 2% (v/v) B-27 (Gibco, 1750444, USA). Briefly, dissected cortex was minced into small pieces and then collected in Leibovitz's Medium

(L15) (Sigma-Aldrich, L5520, Germany) containing 2 mM glutamax (Gibco, 35050061, USA), 100 U/100 µg penicillin/streptomycin, 250 ng amphotericine (Sigma-Aldrich, A5955, Germany), 2% (v/v) B-27 (Gibco, 1750444, USA). 6 U/ml papain (Sigma-Aldrich, P4762, Germany) and 50 µg/ml DNaseI (Sigma-Aldrich, D4513, Germany) were also added and it was incubated at +4ºC for 45 minutes for digestion. After incubation the tissue was gently triturated by pasteur pipette and the sample was centrifuged at 800 rpm for 3 minutes at +4ºC. After discarding the supernatant, the pellet was resuspended in Leibovitz's Medium (L15) (Sigma-Aldrich, L5520, Germany) containing 10 % (v/v) fetal bovine serum (FBS) (Thermo Fisher Scientific, 10500064, USA), 2 mM glutamax (Gibco, 35050061, USA), 100 U/100 µg penicillin/streptomycin, 250 ng amphotericine (Sigma-Aldrich, A5955, Germany), 2% (v/v) B-27 (Gibco, 1750444, USA) and was incubated at room temperature for 10 minutes. After incubation it was re-centrifuged at 800 rpm for 3 minutes at +4ºC and supernatant was discarded. Pelleted cells were resuspended in Neurobasal A medium (Gibco, 10888022, USA) containing 2 mM glutamax (Gibco, 35050061, USA), 100 U/100 µg penicillin/streptomycin, 250 ng amphotericine (Sigma-Aldrich, A5955, Germany), 2% (v/v) B-27 (Gibco, 1750444, USA).

Neurons were cultured on poly-L-lysine (Sigma-Aldrich, P6282, Germany) coated glass-bottomed 35 mm petri dishes and incubated in a cell culture incubator with 5% CO_2 at 37 ºC. After 24 hours incubation, imaging was performed under Primo Vert invert microscop (Zeiss Technologies, Germany) with 20X objective. Cells were seeded into 35 mm petri dishes at a density of $25x10^3$ cells for cell viability experiments. Each experiment was repeated at least three times.

Rifampicin Application

After 24 hours of culturing, three different rifampicin (Sigma-Aldrich, M9511, Germany) concentrations (1 µM, 10 µM and 100 µM) were added to the primary cortical neurons 15 minutes before laser axotomy procedure in order to observe protective effect of rifampicin in these dosages.

Laser Axotomy

In order to cut the axons of cultured primary cortical neurons, PALM Combisystem Microdissection microscobe (Zeiss Technologies, Germany) was used. For axotomy a laser pulse at 337 nm with a 300 lJ energy was applied and the application point was chosen in such a way that the distance of the point from the cell body should be at least two times greater than the diameter of the cell soma. For each experimental group, ten different areas from each petri dish were chosen randomly and thirty neurons were axotomized.

After laser axotomy, cortical neurons were placed into cell culture incubator with 5% CO2 at 37 ºC for 24 hours.

Cell Viability Assay

After 24 hours of incubation of axotomized cells, propidium iodide (PI) (Sigma-Aldrich, P4170, Germany), a nuclear dye labeling only dead cells with red fluorescence, was used to distinguish the axotomized dead cells from axotomized viable cells. Cells were treated with PI at 7.5 µM concentration and the number of dead and live cells were counted using PALM Combisystem Microdissection microscobe (Zeiss Technologies, Germany). Numbers of PI-positive dead cells were counted from chosen areas at each petri dish as mentioned above.

Statistical Analysis

The cell viability data were statistically evaluated by using one-way ANOVA test in SPSS (version 18, IBM, USA) and p values less than 0.05 are considered significant. All values are given as Mean ± Standard deviation (SD).

RESULTS

Cultured primary cortical neurons were visualized in two different magnifications in the light microscope (Figure 1A and 1B). Then, rifampicin was applied in three different concentrations (1 µM, 10 µM and 100 µM) and after 15 minutes of the application laser axotomy was implemented in order to evaluate the possible acute neuroprotective effect of rifampicin (Figure 2). During the axotomy procedure the injury site was chosen according to the criterion mentioned in the previous section.

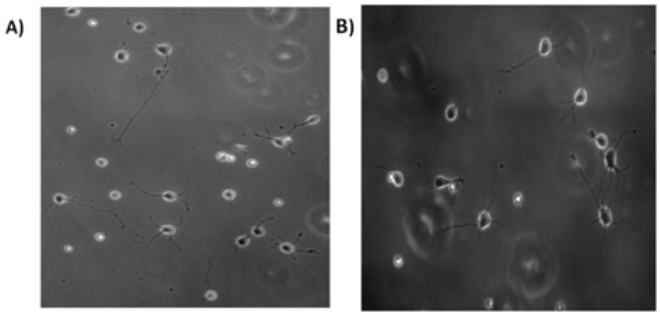

Figure 1. Representative light microscope images of the cultured primary cortical neurons. 20X magnification (A) and 40X magnification (B).

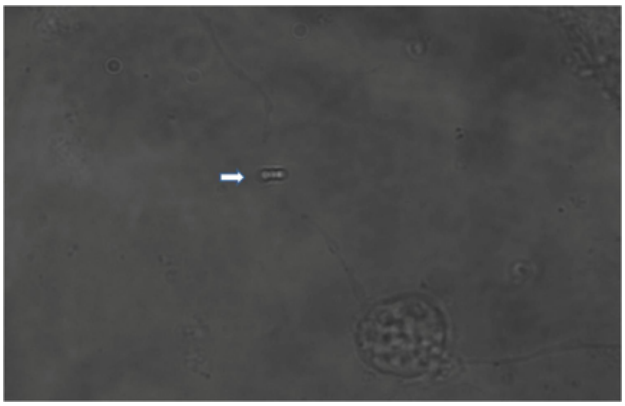

Figure 2. Representative light microscope image of the axotomized primary cortical neuron after rifampicin application. The white arrow indicates the site of laser axotomy. 100X magnification with oil immersion.

Laser axotomy application to the primary cortical neurons resulted in a dramatic reduction of surviving neuron to approximately 80.45% as compared to negative control to which no laser axotomy was applied and no rifampicin was added (Figure 3, $p<0.001$). When rifampicin-treated groups were compared to axotomy-only group, 1 µM rifampicin increased the survival of the axotomized neuron by 87.5% ($p<0.05$), 10 µM increased the survival by 78.95% ($p<0.05$) and 100 µM rifampicin increased the survival by 114.30% ($p<0.05$) (Figure 3).

DISCUSSION

In this study the injured primary cortical neurons were evaluated by measuring their viability after 24 hours. Our model has several unique advantages such as it excludes the possible influences of inflammation and hypoxia and it also provides an isolated experimental setting in which the impact of mechanical injury on cortical neurons can be assessed. This setting also enables us to identify the direct effect of candidate neuroprotective molecules on neurons.

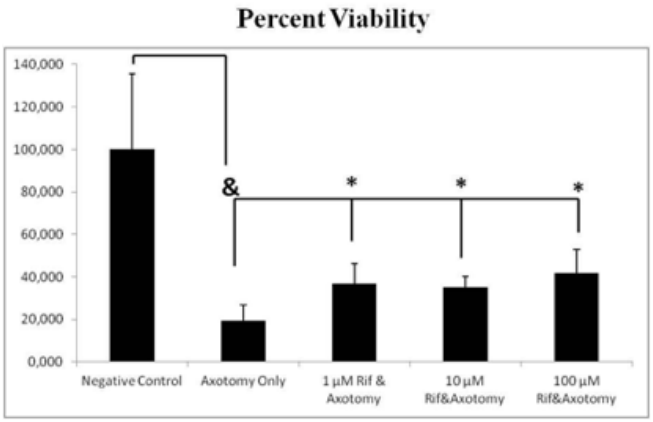

Figure 3. Percent viability of the primary cortical neurons after PI staining. * indicates $p<0.05$ when all three rifampicin groups were compared with the axotomy-only group. & indicates $p<0.001$ when axotomy-only group was compared with the negative control group. Data are given as mean±SD.

Although only the neuronal viability has been investigated, the main goal here was to conduct a preliminary study in order to get reliable data about the laser axotomy if it is a convenient in vitro model for investigating the neuroprotective effect(s) of rifampicin. Our study is the first exhibiting that rifampicin increases neuronal cell survival after laser axotomy. In order to elucidate the acute neuroprotective effect of rifampicin, it was added 15 minutes prior to laser axotomy. We deliberately chose this time point, because 15 minutes duration is the optimal time window for taking up the rifampicin in the in vitro models if its acute effects are investigated.

As it is known that the effect of secondary injury mechanisms initiated by axotomy injury leads to neuronal death within 24 hours of the injury [3], it would be reasonable to consider that rifampicin with its all three dosages reversed the laser axotomy-induced neuronal damage by increasing the survival of the cortical neurons significantly during this time window.

Our results show that there is no linear relationship between the rifampicin concentrations and the percentage of neuronal cell survival. A possible explanation for this situation could be that cellular response to a given drug is not linear generally, and almost all intra- or inter-cellular processes reveal a nonlinear character because of the multi-level and complex relationships present nearly every level of a cell or a multicellular organization.

It is already known from the literature that rifampicin decreases oxidative stress, neutrophilic infiltration and microglial activation. By doing these it has an anti-inflammatory effect in the brain. Rifampicin also increases cholinergic transmission and clearence of amyloid-beta oligomers [19-22]. In our in vitro model, the most probable effect of rifampicin on the neuronal survival would be its free radical scavenging ability.

In clinical studies, the neuroprotective role of rifampicin seems to be controversial. While the Namba et al. shows that rifampicin treatment decreases the amyloid-beta aggregates in non-demented elderly leprosy patients, their results could not be replicated independently [23]. Another study reveals that oral treatment of rifampicin in mild-moderate AD patients improves the cognitive functions of the patients, but the high dose and long duration of the rifampicin treatment for neuroprotection complicate the situation [24,25].

Here in our in vitro experimental setting we demonstrated the possible acute neuroprotective effect of rifampicin and our study would open a new field about the neuroprotective effect of this drug in the context of traumatic conditions such as spinal cord injury or traumatic brain injuries damaging axons.

Limitations of The Study: As an in vitro laser-induced axotomy model using primary cortical neurons, our study reached its goal and it does not have any internal limitations.

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Conflict of Interest: The authors have no conflicts of interest relevant for this article.

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