miRNA-19a exerts an anti-apoptotic effect in spinal cord injured rats via the PTEN pathway

Lu Chen, Xing Li, Jianping Zhu, Binwu Xu, Yurong Gu

The Second Affiliated Hospital of Nanchang University, No. 1 Minde Road, Nanchang, China

Corresponding author:
Dr. Yurong Gu
The Second
Affiliated Hospital
of Nanchang University
No. 1 Minde Road
Nanchang, China
E-mail: ReyFreymom@yahoo.com

Abstract

Introduction: Contusion spinal cord injury is involved in a number of cellular, biochemical and molecular changes. We studied the overall expression pattern of miRNAs on day 1 and 3 after spinal cord injury and the involved pathways.
Material and methods: A spinal cord injury model was developed by contusion injury in rats. Microarray analysis and qRT-PCR were done for expression of miRs. The Basso, Beattie and Bresnahan (BBB) locomotor score was determined after spinal injury. Lesions at the injured site were analyzed by cresyl staining. Western blot analysis was carried out to analyze protein levels. Immunohistochemical staining was done to evaluate immunoreactivity. TUNEL assay was performed to determine the number of apoptotic cells.
Results: The microarray analysis data suggested that about eight miRs were upregulated whereas four were downregulated in rats subjected to spinal cord injury on day 1. On comparing sham operated rats from the day 3 group two miRs were overexpressed and four were downregulated. miR-19a was the most deregulated. miR-19a antagomir was used as an inhibitor, which aggravated the functional deficit, decreased the protection of spinal cord tissue and elevated the number of apoptotic cells. The treatment of miR-19a antagomir increased the expression of FasL along with PTEN, but it failed to affect the levels of PDCD4.
Conclusions: The results suggested that miR-19a plays a potential role in halting the neuronal cell death spinal cord injury and that the protective role of miR-19a may be due to its regulatory effect on pro-apoptotic genes.

Key words: antagomir-19a, apoptosis, FasL, PDCD4, PTEN, spinal cord injury.

Introduction

Spinal cord injury (SCI) is reported to be one of the commonest and most damaging injuries encountered by neurosurgeons. SCI can lead to permanent disability such as loss of sensation, loss of movement and paralysis. Treating SCI remains one of the biggest challenges faced by neurologists. Although many treatment approaches have been explored, they have shown limited success so far [1].

The molecular mechanisms involved in SCI are complicated and involve multiple signaling pathways. The post-traumatic inflammatory response is one of the mechanisms that contribute to progressive tissue damage after SCI. After SCI, inflammatory mediators such as cytokines and eicosanoids accumulate at the injury site, and cells such as mac-

rophages, neutrophils and lymphocytes infiltrate rapidly at the injury site, leading to progression of SCI. Tumor necrosis factor α (TNF-α) is a important mediator of inflammation contributing to cell death in neurodegenerative disorders. Increased levels of TNF-α in the injured spinal cord have been a feature hours after injury [2, 3].

MicroRNAs (miRs) are noncoding, endogenous and single stranded RNAs made up of 22 nucleotides. MicroRNAs are responsible for regulating the expression of a number of genes through RNA-mediated silencing complexes. miRs direct the genes to mRNAs, where they can inhibit the process of translation, causing destructive cleavage [4]. About 6% of the genes are reported to be regulated by miRs [5]. As a result, miRs may significantly affect the signaling inflammatory pathways, leading to pathologies after spinal cord injury. Hence, miRs could become potential and novel targets for treating spinal cord injury.

Evidence suggests that a number of miRs are expressed in the central nervous system (CNS) [6, 7]. Recently a few miRs have been identified to be involved in a number of neurological problems including trauma-mediated CNS injury and neurodegenerative diseases [8–14]. In previous research it was demonstrated that miR-19a is over-expressed and was involved in the regulation of neuropathic pain in rats [8]. In another report, miR-19a was found to be upregulated in ischemic stroke and was also declared to be a novel biomarker [9]. In a study presented by Ge et al., miR-19a attenuated cerebral ischemic injury by targeting Adiponectin receptor protein 2 (ADIPOR2), suggesting it as a novel target for treating ischemic brain injury [10]. In addition, some studies have confirmed the involvement of miRs in the pathogenesis of spinal cord injury in animal models [11–13]. Recently TNF-α, a member of the Fas ligand gene (FasL), has been identified to be a favorable target of miR-19a [14]. In addition, miR-19a has also been found to exert a protective effect on cardiac tissues against hypoxia-mediated injury [15]. However, the role of miR-19a in spinal cord injury remains unexplored. Here we postulate that as miR-19a is a novel biomarker in ischemic stroke and neuropathic pain, it could be an interesting target in spinal cord injury.

In the present work, we performed microarray analysis for the expression of various microRNAs in a rat model of contusion SCI. We found that miR-19a was one of the highly deregulated miRs after SCI. Firstly, it was demonstrated that inhibition of miR-19a using an antagomir aggravated the functional deficit, decreased the protection of spinal cord tissue and elevated the number of apoptotic cells. The pro-apoptotic genes such as PDCD4, FasL and PTEN which are reported to be the direct targets of miR-19a are studied in many disorders and cell types [16–18].

PDCD4 only is defined as Programmed cell death-4; previously it has been reported to be upregulated under apoptotic inducing conditions. Studies have suggested that PDCD4 acts as a tumor suppressor and also as a translation inhibitor. PDCD4 halts the progression and invasion of tumor, and inhibits translation of specific mRNA via binding mRNA molecules and eukaryotic initiation factor 4A. In addition, the expression of PDCD4 is lost or inhibited in some tumors [19]. Fas is expressed by a number of normal cells, such as colon cells. Also these cells are found to be sensitive to Fas-induced apoptosis. Expression of Fas has been reported to be upregulated in a spinal cord injury animal model [20]. PTEN, i.e. phosphatase and tensin homolog, has also been studied extensively. PTEN can lead to dephosphorylation of both inositol lipids and proteins. A study recently suggested that the levels of PTEN were upregulated significantly after the animals were subjected to spinal cord injury [21].

In the present study knockdown of miR-19a in vivo after spinal cord injury upregulated the levels of PTEN and FasL in the cord tissue, but failed to alter the levels of PDCD4. Therefore, the findings of our study suggested that miR-19a can play a potential role in the pathogenesis of SCI.

Material and methods

Experimental animals

For the study, adult male Sprague-Dawley (SD) rats weighing 180–220 g were used. The animals were supplied by the animal house of The Second Affiliated Hospital of Nanchang University, China. All the experimental protocols were approved from the animal ethical committee of The Second Affiliated Hospital of Nanchang University China; the approval number was NU00748A19. The animals were housed in separate cages and maintained under controlled environmental conditions of temperature and humidity; the animals were subjected to a 12 h dark and light cycle and were provided with free access to food and water.

Development of contusion moderate SCI model

The animals were subjected to anesthesia with sodium pentobarbital (40 mg/kg) (9 mg/ml). The rats were subjected to laminectomy at T10 (vertebra 10). The contusion injury (moderate) was done by modified Allen's weight drop instrument; the setup consisted of an eight gram weight kept at a height of 40 mm. The sham operated rats underwent laminectomy and no other procedure.

Experimental design

The study was divided into two parts; in the 1st part of the study miR microarray analysis was done. Microarray analysis was done to examine the patterns of miR expression on the 1st and the 3rd day after SCI. The animals were divided into 4 groups, named the sham-operated group (day 1, i.e. group 1, and day 3, i.e. group 2, $n = 3$/group), SCI induced (day 1, i.e. group 3, and day 3, i.e. group 4, $n = 3$/group). After this, qRT-PCR analysis was done to confirm the results of the microarray study. The rats were again divided into 4 (day 1, i.e. group 1, and day 3, i.e. group 2, $n = 5$/group), SCI induced (day 1, i.e. group 3, and day 3, i.e. group 4, $n = 5$/group).

In the 2nd part of the study, the rats were treated with miR-A antagomir. The SCI induced animals were divided into 2 groups, viz. a negative control group (NC) and the miR-A antagomir treated group. The NC group ($n = 3$) animals were submitted to SCI and were injected with NC antagomir via the intrathecal route 20 nmol/ml). In the 2nd group the SCI subjected rats ($n = 3$) were given miR-A antagomir (20 nmol/ml) for 3 days. After the protocol of 3 days, the rats from both groups were sacrificed (1 from each group) by overdose of sodium pentobarbital (240 mg/ml); the protocol was in accordance with ethical guidelines. Subsequently, the spinal cord was removed and a long segment measuring 10 mm from the region of injury was harvested. The rats were sacrificed based on the different parameters such as lesion identification by cresyl staining and motor function score at 28 days after SCI. Similarly, expression of target proteins such as FasL, PTEN and PDCD4 was determined on day 1 and day 3 of SCI. The qRT-PCR analysis of miR-A was done on the 1st and the 3rd day after SCI. TUNEL staining and in situ hybridization was done on the 1st and the 3rd day after SCI (Figure 1).

MiRNA microarray analysis

For microarray analysis, the rats after 1 or 3 days of laminectomy were subjected to anesthesia, then a piece of spinal cord about 10 mm long which included the injured portion was harvested and was frozen in liquid nitrogen. The spinal tissues were then submitted to isolation of RNA using TRIzol reagent along with the miRNeasy kit following the supplied instructions. The quantitative and qualitative analysis of RNA and miRNA was performed using a spectrophotometer, whereas the RNA integrity was assessed using gel electrophoresis.

After isolating the RNAs from the spinal tissues, miR labeling was done using the Label IT miRNA Labeling Kit (Mirus Bio, USA) following the manufacturer's instructions. About 1 µg of each sample was labeled in the 3′ region using the fluorescent labeling reagent from the kit following the supplied instructions. Briefly, 2 µl of aqueous RNA solution was added with 1 µl of alkaline phosphatase and calf intestinal buffer (CIP buffer). The resultant mixture was incubated for 25 min at room temperature followed termination by incubating for 5 min at 95°C. After this about 3 µl of labeling buffer along with 1.5 µl of fluorescent label, 2 µl DMSO and 2 µl of labeling enzyme were added together in the mixture. The final mixture was incubated for 1 h at 16°C followed by termination by incubating for 20 min at 65°C.

After the labeling process was completed, the fluorescent labeled samples were subjected to hybridization on an LNA array following the supplied instructions. After the process of hybridization, the slides were collected, rinsed many times using washing buffer and finally were dried in a high speed centrifuge for 5 min at 500 rpm. The slides were scanned and the images were processed for grid alignment followed by extraction of data using GenePix software. For evaluating the normalization factor, the average of replicated miRs was done to calculate the normalization factor.

Administration of miR-19a antagomir and negative control antagomir

The miR-19a antagomir was infused in SCI rats by a catheter using a minipump immediately after inducing spinal cord injury. Briefly, both the minipump and the catheter were implanted by surgery just after the SCI. The miR-19a antagomir and negative control was solubilized in
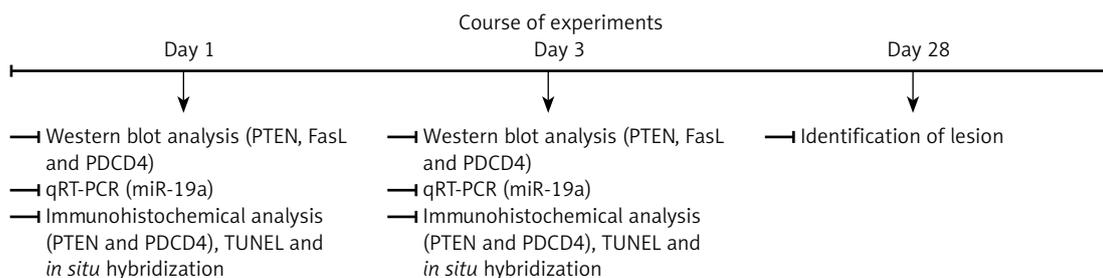

Figure 1. Experimental design for the study for 28 days

0.9% saline solution having the concentration 20 nM/ml; the resultant solution was filled in osmotic minipumps followed by continuous infusion in the spinal cord of SCI rats (1 μl/h). After implanting the catheter, surgery was done at the T12 and T13 rat vertebrae. A 32 number catheter was introduced via the dural incision. The catheter was joined to a 2 cm piece of PE10 tubing, which was attached to the osmotic pump. The osmotic pumps in the rats were implanted subcutaneously. Each rat was injected with an intramuscular injection of penicillin (40000 U) during the surgery followed by a once a day dose for the next 5 days. The rats were kept in polypropylene cages and were provided a normal diet along with free access to water.

Locomotor activity by Basso, Beattie and Bresnahan (BBB) locomotor score

The locomotor activity was evaluated by the Basso, Beattie and Bresnahan (BBB) locomotor score. The activity was assessed on the 1st, 3rd, 7th, 14th, 21st, and the 28th day after the SCI induction. The score measured the locomotor activity for 4 min. The score was recorded and compared to the scales provided earlier.

Identification of lesions by cresyl staining method

At the end of the last behavioral test done on the 28th post-surgery day, the rats were euthanized using overdose of sodium pentobarbital (240 mg/ml). The rats were perfused using isotonic solution of sodium chloride (0.9% w/v of NaCl) followed by addition of 500 ml of paraformaldehyde (PFA) (Sigma Aldrich USA) (4%) in 0.1 M PBS (pH 7.4). The injured site and a 1 cm portion of spinal cord was dissected and fixed in fixing media for 24 h at 4°C. The tissues after fixing were imbedded in paraffin. From the paraffin embedded a section of 10 μM thickness was obtained and mounted on slides. The obtained tissue section was stained with cresyl-violet solution (Sigma Aldrich USA) (0.5%) and viewed under a microscope (Olympus, NY). The lesion areas and the spared tissue areas in the spinal cord tissue sections were identified using Image pro software (Media, USA). The spared tissue area was the part of spinal cord section which showed normal anatomical structure, whereas the area with a low percentage of spared tissue was determined as the injured epicenter.

Quantitative real-time PCR (qRT-PCR)

The injured epicenter of spinal cord was processed and total RNA was isolated using TRIzol re-agent. The obtained total RNA from each sample was subjected to reverse transcription to cDNA using a PrimeScript kit (TaKaRa, Japan) followed by qRT-PCR analysis using SYBR primer Ex Taq and miR-19a specific primers. The expression of miRNA levels was normalized to U6 (a small nuclear RNA (snRNA)) expression for each sample; the $2^{\Delta\Delta Ct}$ method was used for gene expression.

Western blot analysis

The animals were subjected to euthanasia by overdose of sodium pentobarbital (240 mg/ml) divided into day 1 and 3 after injury. The spinal cords were harvested and a segment measuring 1 cm with the injured site was used for studying the expression of proteins by western blot. The harvested spinal tissues were homogenized and subjected to lysis, and total protein was determined using protein estimation kit (Sigma Aldrich USA). About 20 μg of protein was submitted to electrophoresis on sodium dodecyl sulfate/polyacrylamide gel (12%) and then transferred to PVDF membranes (Thermo Fisher USA). The PVDF membranes were blocked using 5% nonfat milk for 1 h. The membranes were incubated for 12 h at 4°C along with rabbit anti-FasL antibody (1 : 1000, Abcam USA), rabbit anti-PTEN antibody (1 : 1000, Abcam USA), rabbit anti-PDCD4 antibody (1 : 1000 Abcam USA) and β-actin (1 : 1000, Abcam, USA). The membranes were then incubated with horseradish peroxidase conjugated IIry antibody (1 : 10000) for 60 min at 37°C. The proteins were viewed using enhanced chemiluminescence; β-actin was used as loading control.

Immunohistochemical staining

The rats were subjected to euthanasia as described earlier on day 1 and 3 after spinal cord injury (n = 3/group). A segment of spinal cord with the injured site measuring 10 mm was harvested. The isolated spinal tissue was fixed and embedded in paraffin, and sections of 5 μm thickness were obtained. The sections were deparaffinized with xylene and hydrated with alcohol. The unmasking of epitope was microwave assisted in 10 mM citrate buffer at pH 6 at 800W for 3 min followed by cooling for 30 min. The endogenous peroxidase was inactivated by incubating for 10 min in 3% H_2O_2 at room temperature. The sections were washed at least 2 times with phosphate buffer saline (0.01M) and were blocked using 10% fetal bovine serum for 30 min followed by incubation for 12 h at 4°C along with Iry antibodies named anti-PTEN (1 : 1000), anti-FasL (1 : 1000) and anti-PDCD4 (1 : 1000). The tissue sections were then incubated with IIry antibodies (IgG antibody). Diaminobenzidine (DAB) staining was car-

ried out to determine the immunoreactivity. The slides were covered with a cover slip and were analyzed under a light microscope.

TUNEL assay

The prepared slides of spinal tissues were used for studying neuronal cell apoptosis by TUNEL assay. The TUNEL staining was done using a TUNEL assay kit (Abcam, USA). The sections were dewaxed by xylene and then rehydrated using alcohol; the sections were placed in water. The processed spinal tissue sections were incubated for 10 min with 20 µg/ml proteinase K working solution at room temperature. The slides were washed 3 times with phosphate buffer saline before incubating with TUNEL mixture for 60 min at room temperature. The sections were washed with phosphate buffer saline and incubated with HRP-streptavidin reagent (1 : 200) in phosphate buffer saline for 30 min at 37ºC. The sections were washed again with phosphate buffer saline 3 times and the sections were incubated with a solution mixture of H_2O_2 (0.03%) and DAB (0.04%) for 10 min. The sections were again washed with phosphate buffer saline and then counterstained with hematoxylin. The sections were washed 2 times with de-ionized water and mounted on slides with a cover slip. The TUNEL positive cells were quantified from each section twice using a light microscope. Briefly, 2 spinal cord tissue sections were chosen for each animal, 5 areas were randomly selected and the cell counts were done manually; positive cells were calculated.

In situ hybridization

For *in situ* hybridization, the slides were prepared as described earlier. Briefly, the tissue sections were dewaxed by xylene and rehydrated in alcohol and exposed to an aqueous solution of diethyl pyrocarbonate (DEPC). Inactivation of endogenous peroxidase was done by incubating the sections in H_2O_2 (3%) for 10 min. The tissue sections were then treated with proteinase K for 25 min and were washed with sodium chloride Tris buffer and fixed using PFA (4%) for 15 min. The slides were again washed with phosphate buffer saline, and the slides were blocked with hybridization buffer (Thermo Fisher USA). The slides were washed after the process of hybridization and incubated with HRP conjugated anti-DIG antibody. The sections were washed with phosphate buffer saline 3 times. DAB was used to visualize peroxidase staining. The sections were washed again with phosphate buffer saline 3 times and stained with hematoxylin. The cells were counted manually in 5 random areas of sections; positive cells were visualized.

Statistical analysis

The data were analyzed using GraphPad software. Analysis of variance (ANOVA) was performed to compare BBB scores. The other results were tested by Student's *t* test. All the data are presented as mean ± SEM; values < 0.05 were considered as significant.

Results

Abnormal expression of miRNAs in injured spinal cord

To find the potentially involved miRs in spinal cord injury, we employed microarray analysis to trace the levels of miRs in spinal cord tissues subjected to contusion spinal cord injury. The results suggested that, compared to sham operated rats, about 8 miRs were upregulated whereas 4 were downregulated in rats subjected to spinal cord injury in the day 1 group (Figure 2 A). On comparing sham operated rats in the day 3 group two miRs were overexpressed and four were downregulated in spinal cord injured rats (Figure 2 A). Among the identified miRs, miR-23, miR-24a, miR-126 and miR-19a were abnormally expressed; these miRs were further subjected to qRT-PCR analysis (Figure 2 B). From these miRs, the microarray and RT-PCR analysis suggested that miR-19a was the most deregulated; also miR-19a has been reported to exert protective action in many models of injury [10–13, 15]. Hence, we targeted our study on miR-19a in spinal cord injury for further study.

miR-19a antagomir exerts an inhibitory effect on miR-19a

To determine the inhibitory effect of miR-19a antagomir on expression of miR-19a *in vivo*, we did *in situ* hybridization using an LNA-containing antisense oligonucleotide probe followed by qRT-PCR analysis. We found that miR-19a was present in the nuclei of many cells compared to the negative control (Figure 3). The miR-19a antagomir decreased the count of miR-19a positive cells in day 1 and 3 groups of rats (Figures 3 A, B, D). The results of qRT-PCR were in parallel to findings of *in situ* hybridization and suggested that levels of miR-19a decreased significantly in day 1 and day 3 groups in RNAs of rats treated with miR-19a antagomir compared to the negative control (Figure 3 C).

Inhibition of miR-19a aggravated the functional deficit mediated by spinal cord injury

The rats after contusion spinal cord injury were paralyzed from both the hind limbs. The rats showed recovery of spontaneous function after spinal cord injury in day 1 and day 3 groups (Fig-
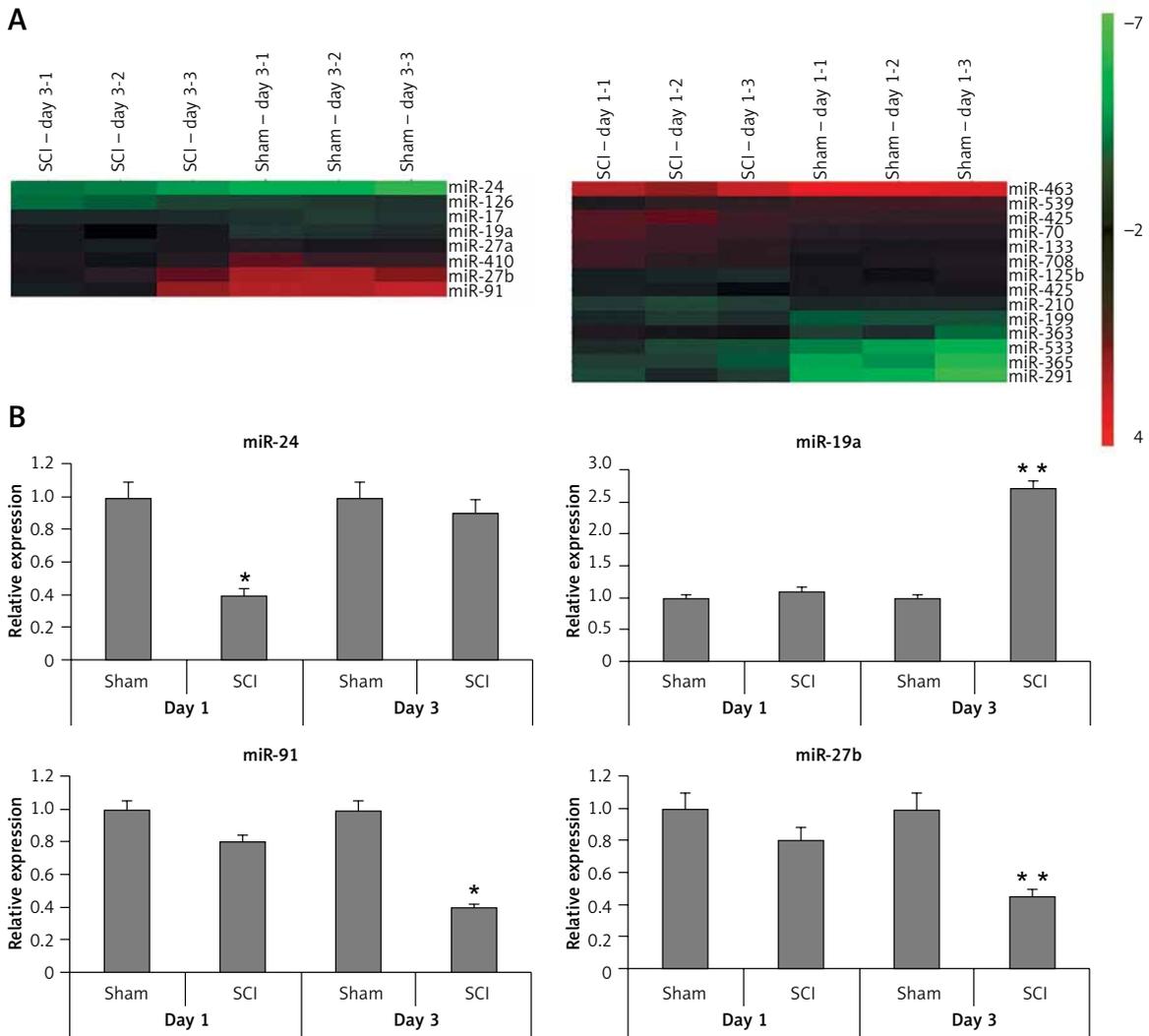
Figure 2. Cluster analysis (heat map) for alterations in expression of miRNAs after spinal cord injury. **A** – Cluster analysis shows expression of differentially expressed miRs in sham operated as well as in spinal cord injured rats, 3 days after injury (*n* = 3 in each group). Cluster analysis showing differential expression of miRs in sham operated and spinal cord injured group of rats on day 1 after injury (*n* = 3 in each group). The color pattern in the heat map is linear: the green color represents the lowest whereas the red shows the highest; color green to red represents upregulated miRs whereas color red to green indicates down-regulated miRs. **B** – Results of qRT-PCR analysis confirmed the results of microarray analysis in rats after day 1 and 3 after spinal cord injury. The results were compared to the sham operated group (day 1 and day 3 post injury)

*P < 0.05 and **p < 0.01.

ure 4). We observed that the hind limb locomotor improved during the experimental period as seen by the increase in BBB score in both the groups. The treatment of miR-19a antagomir attenuated the injury after the 14th day along with the negative control group. After 4 weeks of spinal cord injury, the BBB scores suggested that miR-19a antagomir exacerbated the movement of animals subjected to spinal cord injury.

Inhibition of miR-19a aggravated tissue damage in the injured spinal cord

To evaluate the lesion size, cresyl violet staining was done for spared spinal tissue followed by behavioral studies after 28 days of spinal injury (Figure 5). The sections from the thoracic region of the spinal cord were evaluated; the injured area from the total area was compared for each tissue section (Figure 5 A). The study of sections suggested that the miR-19a antagomir treated rats showed a large lesion not only in the injured area, but also in the area stretching from the epicenter in both the caudal and rostral direction. The miR-19a antagomir treated rats when compared to negative control rats showed a significantly small spared tissue area around the lesion epicenter in the caudal and rostral direction (Figures 5 B, C).
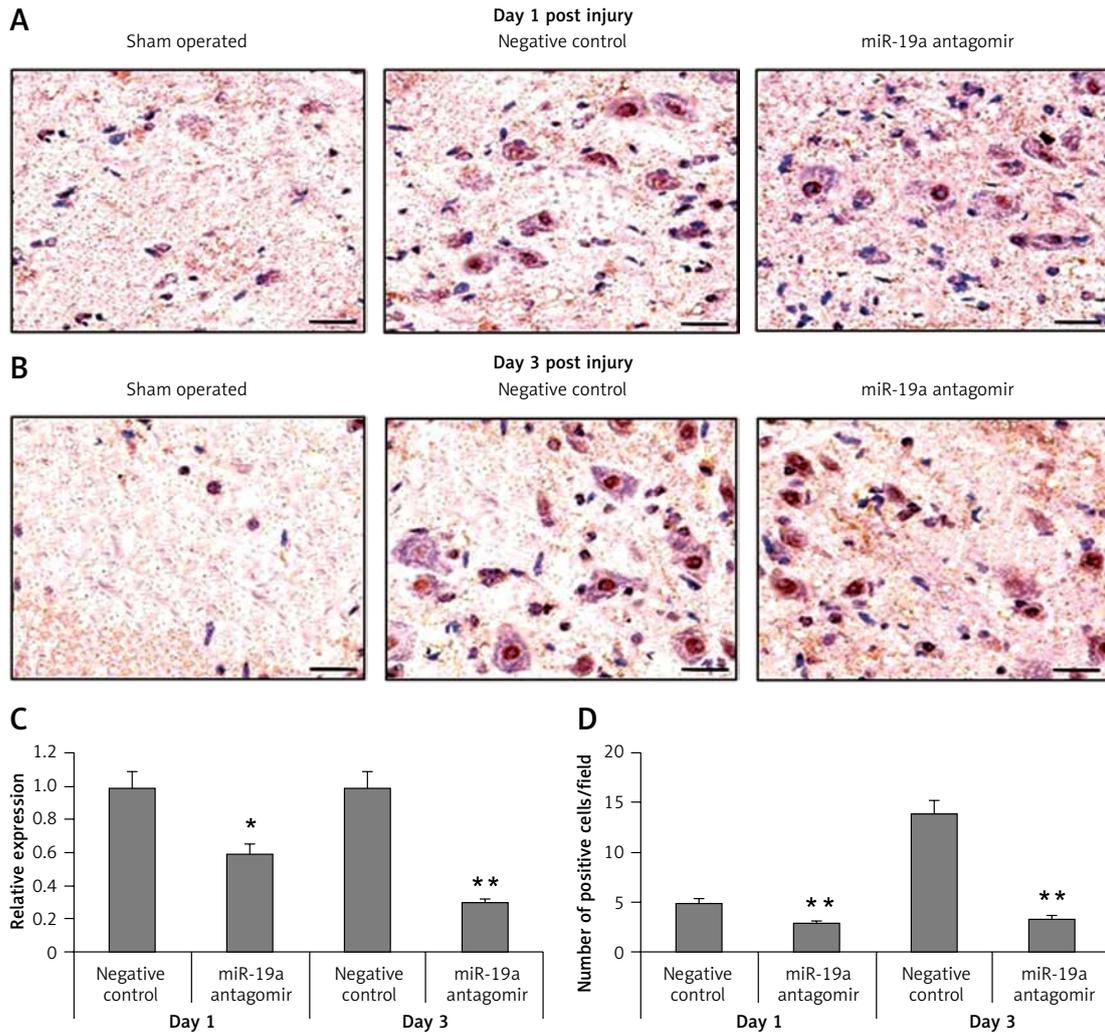
A

Day 1 post injury

| Sham operated | Negative control | miR-19a antagomir |

B

Day 3 post injury

| Sham operated | Negative control | miR-19a antagomir |

C

D

Figure 3. miR-19a antagomir blocks the *in vivo* expression of miR-19a after spinal cord injury. The results of qRT-PCR and situ hybridization for expression of miR-19a after 1 and 3 days after spinal cord injury. miR-19a was localized in the nucleus of cells after spinal cord injury. The miR-19a antagomir decreased the number of miR-19a positive cells significantly as compared to the negative control group after 1 day (**A**, **D**) and 3 days after spinal cord injury. The findings of in situ hybridization and qRT-PCR were parallel to each other and showed that expression of miR-19a decreased significantly on day 1 and day 3 in rats subjected to spinal cord injury and treated with miR-19a antagomir compared to the negative control group (**B**, **D**)
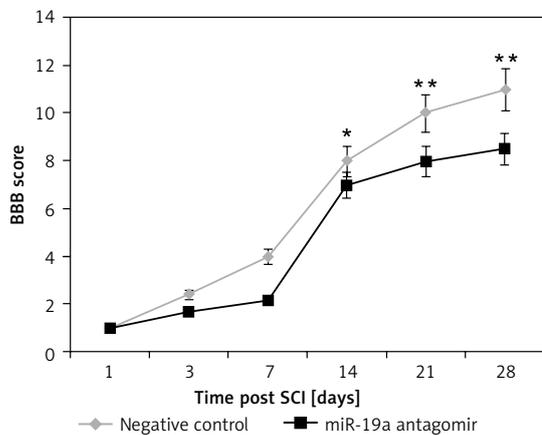
*P < 0.05, **p < 0.01.

Figure 4. Blockade of miR-19a aggravated the functional deficit after spinal cord injury in rats. The hindlimb was assessed using Basso, Beattie, and Bresnahan (BBB) Score for recovery from the 1st day to the 28th day after spinal cord injury. The dysfunctioning of hindlimb was aggravated when treated with miR-19a antagomir compared to the negative control

*P < 0.05; **p < 0.01.
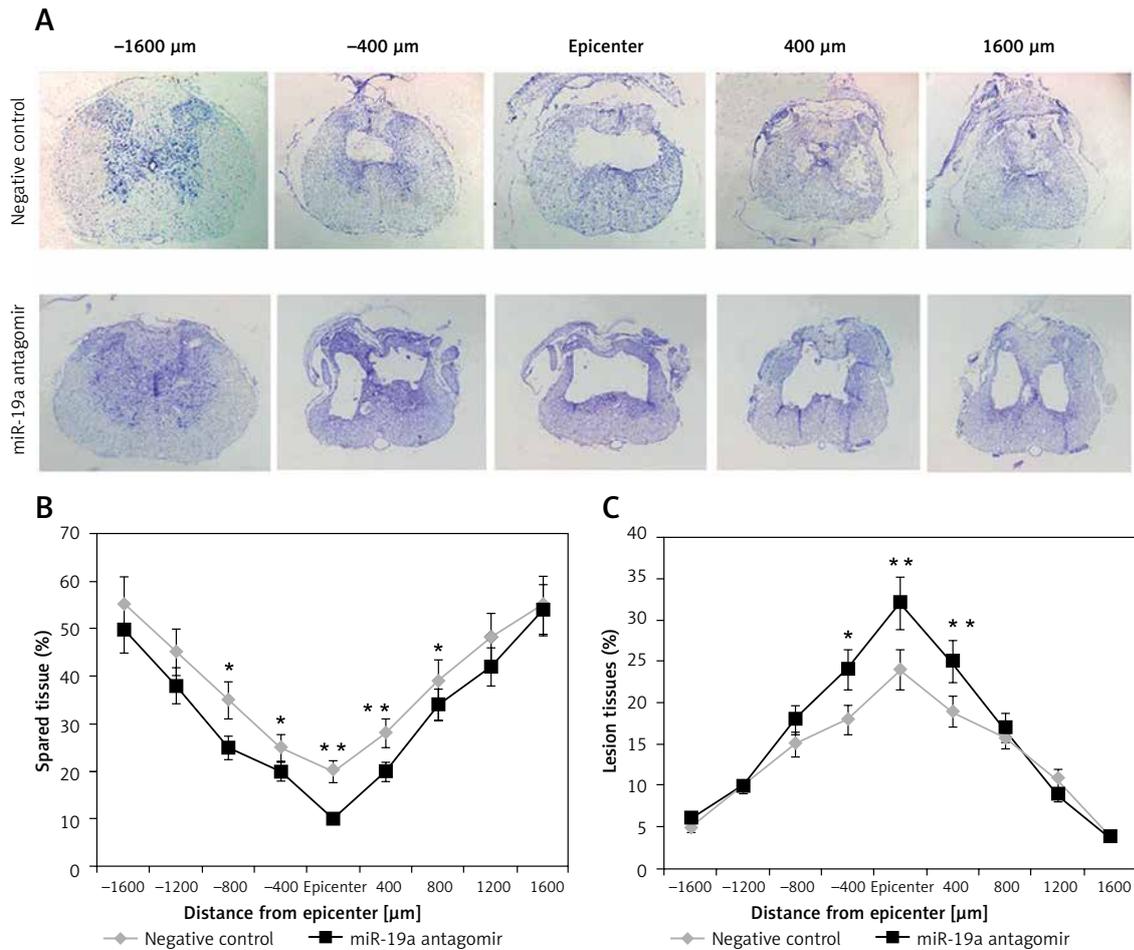
A

B

C

Figure 5. Blockade of miR-19a exacerbates the tissue damage of spinal tissues in spinal cord injured rats. **A** – The spinal cord sections were stained with cresyl violet and were evaluated for size of lesion and spared tissue after 28 days after spinal injury. The sections show spared tissue and lesion size from the injured site and the area 1600 μm rostral and caudal to the injured site after 28 days of injury. **B** – The results of quantitative analysis of spared tissue around the injured site and 1600 μm rostral and caudal to the epicenter after 28 days of injury. **C** – Quantitative analysis of lesion size within the injured area and 1600 μm rostral and caudal to the epicenter after 28 days of injury

*$P < 0.05$; **$p < 0.01$.

Downregulated expression of miR-19a increased apoptotic cells after spinal cord injury

TUNEL staining assay was done to study the effect of miR-19a on apoptotic cells after spinal cord injury. Quantification was done after day 1 and day 3 after injury. We noted that most of the TUNEL positively stained cells were present in the gray matter and a few of the TUNEL positive cells were observed adjacent to white matter. The number of positive stained cells was high in the spinal cord tissue section observed in day 3 post-injury rats and the negative control group. When compared to negative control group rats, the miR-19a antagomir treated rats at day 3 after injury showed a significant increase in the number of TUNEL positive cells compared to the negative control (Figure 6).

Blockade of miR-19a did not alter expression of PDCD4 but increased levels of FasL and PTEN *in vivo*

PDCD4, FasL and PTEN were the established targets of miR-19a; hence we targeted these genes and postulated that they may be regulated by miR-19a *in vivo*. Expression of PTEN, FasL and PDCD4 was evaluated by western blot analysis. It was observed that treatment of miR-19a antagomir compared to the negative control resulted in a significant increase in expression of FasL at the 3rd day after injury, but the levels of FasL were the same on the 1st day compared to the negative control (Figure 7). Similarly, treatment with miR-19a antagomir elevated the levels of PTEN compared to the negative control group on the 3rd day, but no significant changes were seen on the 1st day in both groups (Figures 8 C, D). The results
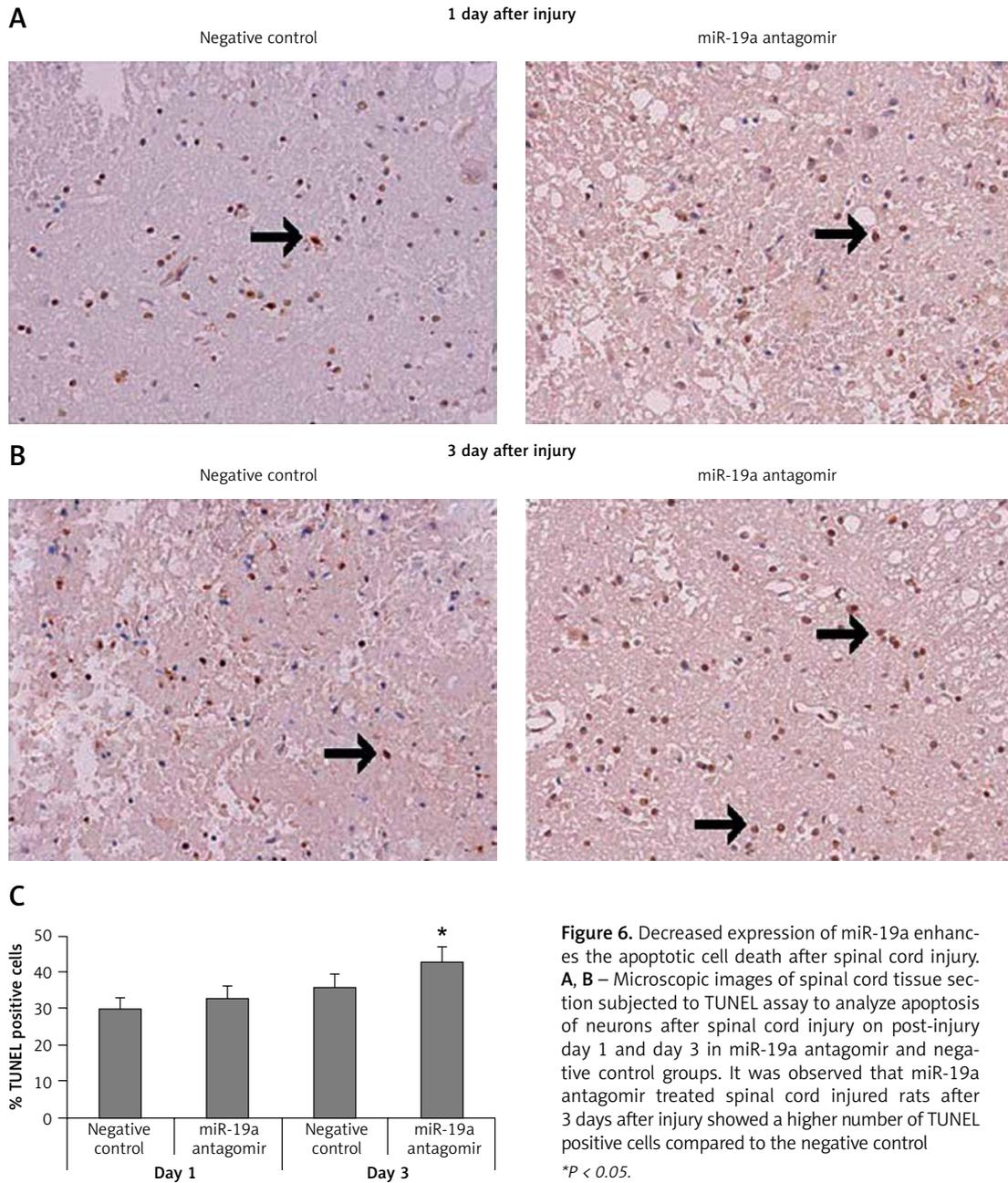
A

1 day after injury

Negative control

miR-19a antagomir

B

3 day after injury

Negative control

miR-19a antagomir

C

Figure 6. Decreased expression of miR-19a enhances the apoptotic cell death after spinal cord injury. **A, B** – Microscopic images of spinal cord tissue section subjected to TUNEL assay to analyze apoptosis of neurons after spinal cord injury on post-injury day 1 and day 3 in miR-19a antagomir and negative control groups. It was observed that miR-19a antagomir treated spinal cord injured rats after 3 days after injury showed a higher number of TUNEL positive cells compared to the negative control
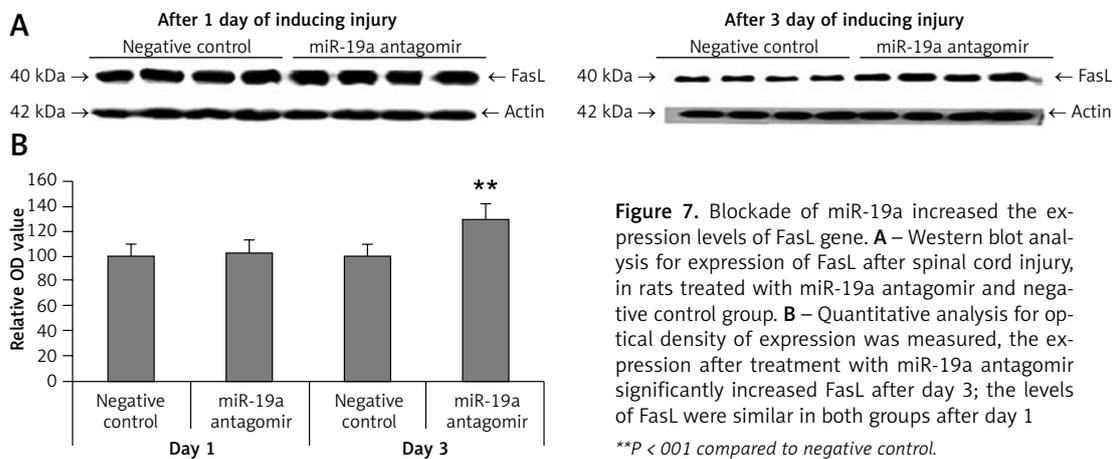
*P < 0.05.

A

After 1 day of inducing injury

Negative control   miR-19a antagomir

40 kDa → ← FasL

42 kDa → ← Actin

After 3 day of inducing injury

Negative control   miR-19a antagomir

40 kDa → ← FasL

42 kDa → ← Actin

B

Figure 7. Blockade of miR-19a increased the expression levels of FasL gene. **A** – Western blot analysis for expression of FasL after spinal cord injury, in rats treated with miR-19a antagomir and negative control group. **B** – Quantitative analysis for optical density of expression was measured, the expression after treatment with miR-19a antagomir significantly increased FasL after day 3; the levels of FasL were similar in both groups after day 1
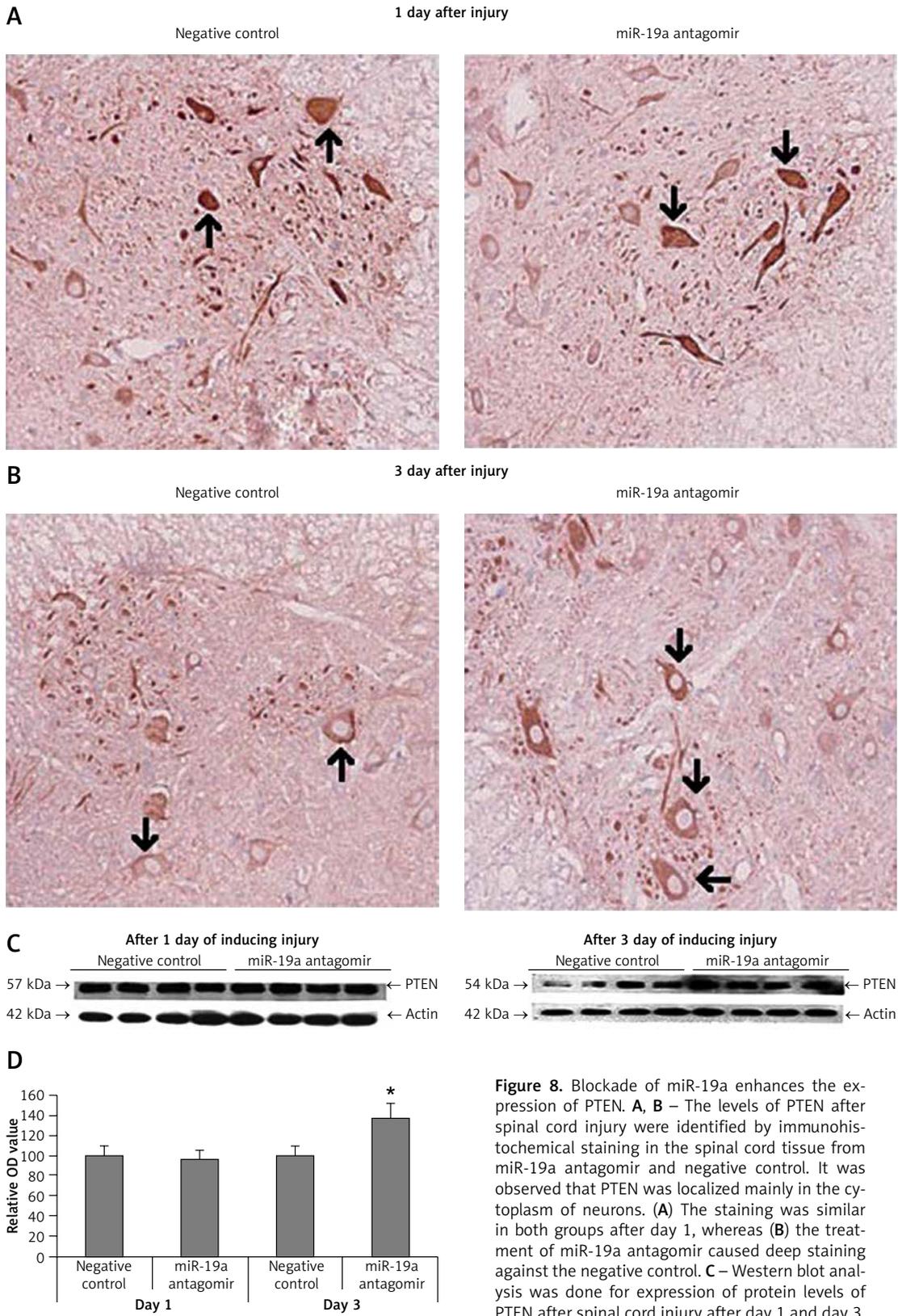
**P < 001 compared to negative control.

Lu Chen, Xing Li, Jianping Zhu, Binwu Xu, Yurong Gu

A

1 day after injury

Negative control | miR-19a antagomir

B

3 day after injury

Negative control | miR-19a antagomir

C

After 1 day of inducing injury

Negative control | miR-19a antagomir

57 kDa → ← PTEN
42 kDa → ← Actin

After 3 day of inducing injury

Negative control | miR-19a antagomir

54 kDa → ← PTEN
42 kDa → ← Actin

D

Figure 8. Blockade of miR-19a enhances the expression of PTEN. **A**, **B** – The levels of PTEN after spinal cord injury were identified by immunohistochemical staining in the spinal cord tissue from miR-19a antagomir and negative control. It was observed that PTEN was localized mainly in the cytoplasm of neurons. (**A**) The staining was similar in both groups after day 1, whereas (**B**) the treatment of miR-19a antagomir caused deep staining against the negative control. **C** – Western blot analysis was done for expression of protein levels of PTEN after spinal cord injury after day 1 and day 3. **D** – The expression was determined by densitometry analysis. The treatment with miR-19a antagomir enhanced the expression of PTEN compared to the negative control group after day 3; no changes were seen after day 1

$P < 0.05$ compared to negative control.

A

1 day after injury

Negative control   miR-19a antagomir

B

3 day after injury

Negative control   miR-19a antagomir

C

After 1 day of inducing injury

Negative control   miR-19a antagomir

62 kDa →   ← PDCD4
42 kDa →   ← Actin

After 3 day of inducing injury

Negative control   miR-19a antagomir

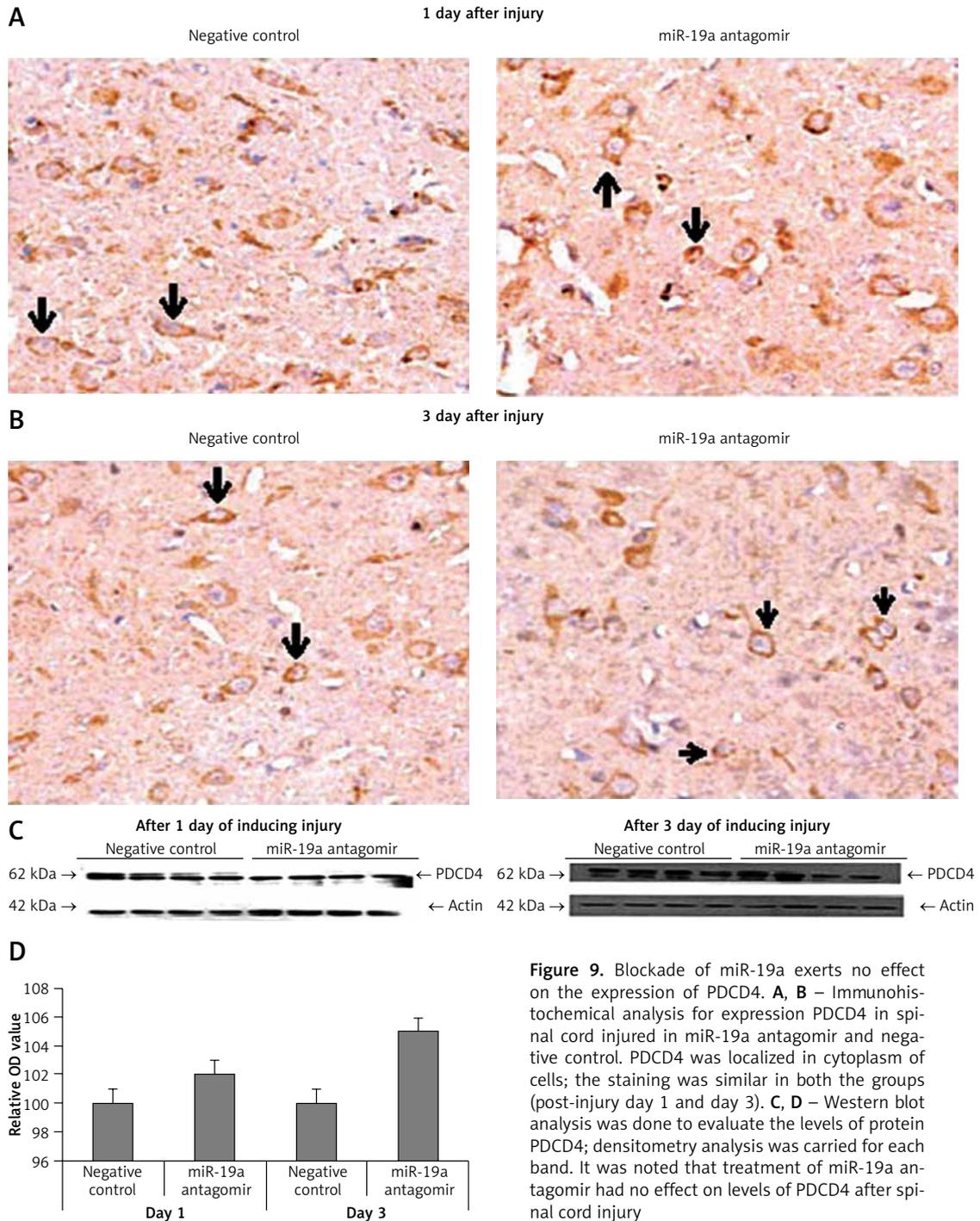62 kDa →   ← PDCD4
42 kDa →   ← Actin

D

Figure 9. Blockade of miR-19a exerts no effect on the expression of PDCD4. **A, B** – Immunohistochemical analysis for expression PDCD4 in spinal cord injured in miR-19a antagomir and negative control. PDCD4 was localized in cytoplasm of cells; the staining was similar in both the groups (post-injury day 1 and day 3). **C, D** – Western blot analysis was done to evaluate the levels of protein PDCD4; densitometry analysis was carried for each band. It was noted that treatment of miR-19a antagomir had no effect on levels of PDCD4 after spinal cord injury

of immunohistochemical staining suggested that PTEN was localized in cytoplasm and upregulated after treatment with miR-19a antagomir on the 3rd day, the expression was parallel in both the groups on the 1st day, and the results were parallel to the findings of western blot. The results also showed that treatment with miR-19a antagomir had no any affect on the expression of PDCD4 after spinal cord injury (Figures 9 C, D). The results of immunohistochemical analysis suggested that PDCD4 was localized in the cytoplasm region, whereas the expression of PDCD4 was identical at the 1st and the 3rd day; the findings were parallel to the outcomes of western blot analysis.

Discussion

Spinal cord injury results in major biochemical and molecular changes which include alterations in expression of mRNA such as *gadd45a, c-myc, cyclin D1* and *cdk4, pcna, cyclin G, Rb,* and *E2F5.* The genes associated inflammation, oxidative

stress, DNA damage, and cell cycle are also found to be altered [16, 17]. Several studies have been done to increase our understanding of expression of mRNA and involved proteins following spinal cord injury and how they correlate with the pathophysiology of spinal cord injury. Recently, researchers have demonstrated that individual miRs can modulate numbers of genes simultaneously by targeting mRNAs and inhibit translation [18]. Hence, alterations in expression of miRs may affect the pathophysiology of spinal cord injury. By performing miRNA microarray analysis, it was found that miR-23, miR-24a, miR-126 and miR-19a were aberrantly expressed in spinal cord injured rats; the results were further confirmed by qRT-PCR analysis. It is to be noted that a number of studies have emerged which have demonstrated aberrant expression of miR-19a in animal models of injury. In a study, miR-19a was found to be over-expressed in ischemic stroke [9]. In a study by Ge *et al.*, miR-19a attenuated cerebral ischemic injury by targeting ADIPOR2, identifying it as a novel target for treating ischemic brain injury [10]. Findings also have confirmed that expression of a number of miRs was altered in the hippocampus region of the brain after traumatic brain injury [22]. Similar to this, our results demonstrated that miR-19a was one of the aberrantly expressed miRNAs after spinal cord injury. miR-19a has been reported to show a potential anti-apoptotic effect in cardiomyocytes and has been found to protect cardiomyocytes from hypoxia-induced apoptosis through the PTEN/p-Akt pathway [23]. In the present study, we found that expression of miR-19a was significantly upregulated in rats after 3 days of spinal injury, inhibition of miR-19a aggravated the tissue damage and functional deficit and enhanced the apoptotic cell death mediated by spinal cord injury. These findings indicate that miR-19a exerts a protective effect in spinal cord injury via inhibiting the cell death of neurons.

miR-19a demonstrated its anti-apoptotic effect by targeting the pro-apoptotic genes. Fas ligand (FasL) is a type-2 category transmembrane protein which belongs to the TNF-α. TNF-α is identified to be one of the potential targets of miR-19a. miR-19a, which belongs to the miR17-92 cluster, has been shown to decrease the expression of FasL in ischemic neural progenitor cells [24]. Overexpression of miR-19a in an animal model inhibited the ischemia-mediated upregulation of FasL, and attenuated apoptosis and heart failure [20]. It has already been evidenced that in many neurological disorders, expression of FasL is upregulated [25–27]. In addition, FasL has a major role in apoptotic cell death after spinal cord injury [25, 27]. Our results suggested that inhibition of expression of miR-19a in an animal model upregulated the expression of FasL. Also we found that miR-19a played an anti-apoptotic role via targeting FasL after spinal cord injury.

PTEN is identified to be one of the potential targets of miR-19a [15]. miR-19a-induced regulation of PTEN has been demonstrated in malignancies such as hepatic cancer, ovarian cancer and myeloma [28–30]. Studies also have established that inhibition of PTEN led to development of uninjured axons in the corticospinal tract, and also led to successful regeneration of injured axons in spinal cord lesion after spinal cord injury [31]; this also increased the survival of neurons [32, 33]. Our findings demonstrated that inhibiting the expression of miR-19a elevated the expression of PTEN and increased the size of the lesion and decreased the spared tissue. The findings suggested that miR-19a may promote generation of neurons and increase the survival of neurons via targeting PTEN after spinal cord injury.

PDCD4 is another important target of miR-19a. PDCD4 is identified to be a tumor suppressor and is found to be downregulated in number of malignancies and is overexpressed during the process of apoptosis [34–36]. PDCD4 is also upregulated in a number of diseases and cell types. Studies have demonstrated that expression of PDCD4 is elevated after inhibition of miR-19a, and miR-19a has a direct binding site on the 3′-UTR region of PDCD4 [33, 37, 38]. Negative regulation of PDCD4 via miR-19a resulted in a protective effect against ischemia-mediated reperfusion injury in heart [23]. In our study, we found that levels of PDCD4 were not altered after inhibiting the expression of miR-19a. The results suggest that though miRs may have different and numerous targets, their modulation may depend or vary based upon the tissue or cell specificity.

In conclusion, the study concludes that spinal cord injury may show a varied pattern of expression of miRs. Among them we found that miR-19a was aberrantly expressed in rats submitted to contusion spinal cord injury. Inhibition of miR-19a in rats aggravated the functional deficit, led to tissue damage and increased apoptotic cell death in animal models of spinal cord injury. We also confirmed that miR-19a possibly exerts its anti-apoptotic effect via targeting the two pro-apoptotic genes PTEN and FasL. These findings suggested that miR-19a may play a crucial role in pathophysiology of spinal cord injury. As miRs are highly unpredictable regulators having an impact on a number of target genes, the exact role of miR-19a in spinal cord injury involving other models needs to be explored.

Conflict of interest

The authors declare no conflict of interest.

R e f e r e n c e s

1. Thuret S, Moon LD, Gage FH. Therapeutic interventions after spinal cord injury. Nat Rev Neurosci 2006; 7: 628-43.
2. Carlson GD, Gorden C. Current developments in spinal cord injury research. Spine J 2002; 2: 116-28.
3. Dumont RJ, Okonkwo DO, Verma S, et al. Acute spinal cord injury, part I: pathophysiologic mechanisms. Clin Neuropharmacol 2001; 24: 254-64.
4. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 2004; 116: 281-97.
5. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 2009; 19: 92-105.
6. Bak M, Silahtaroglu A, Møller M, et al. MicroRNA expression in the adult mouse central nervous system. RNA 2008; 14: 432-44.
7. Kosik KS. The neuronal microRNA system. Nat Rev Neurosci 2006; 7: 911-20.
8. Wang C, Jiang Q, Wang M, Li D. MiR-19a targets suppressor of cytokine signaling 1 to modulate the progression of neuropathic pain. Int J Clin Exp Pathol 2015; 8: 10901-7.
9. Eyileten C, Wicik Z, Rosa SD, Mirowska-Guzel D, et al. MicroRNAs as diagnostic and prognostic biomarkers in ischemic stroke – a comprehensive review and bioinformatic analysis. Cells 2018; 7: 249.
10. Ge XL, Wang JL, Liu X, Zhang J, Liu C, Guo L. Inhibition of miR-19a protects neurons against ischemic stroke through modulating glucose metabolism and neuronal apoptosis. Cell Mol Biol Lett 2019; 24: 37.
11. Liu NK, Wang XF, Lu QB, Xu XM. Altered microRNA expression following traumatic spinal cord injury. Exp Neurol 2009; 219: 424-9.
12. Strickland ER, Hook MA, Balaraman S, Huie JR, Grau JW, Miranda RC. MicroRNA dysregulation following spinal cord contusion: implications for neural plasticity and repair. Neuroscience 2011; 186: 146-60.
13. Yunta M, Nieto-Diaz M, Esteban FJ, et al. MicroRNA dys regulation in the spinal cord following traumatic injury. PLoS One 2012; 7: e34534.
14. Liu M, Wang Z, Yang S, et al. TNF-alpha is a novel target of miR-19a. Int J Oncol 2011; 38: 1013-22.
15. Sun G, Lu Y, Li Y, et al. miR 19a protects cardiomyocytes from hypoxia/reoxygenation-induced apoptosis via PTEN/ PI3K/p-Akt pathway. Biosci Rep 2017; 37: BSR20170899.
16. Di Giovanni S, Knoblach SM, Brandoli C, Aden SA, Hoffman EP, Faden AI. Gene profiling in spinal cord injury shows role of cell cycle in neuronal death. Ann Neurol 2003; 53: 454-68.
17. De Biase A, Knoblach SM, Di Giovanni S, et al. Gene expression profiling of experimental traumatic spinal cord injury as a function of distance from impact site and injury severity. Physiol Genomics 2005; 22: 368-81.
18. Baek D, Villen J, Shin C, Camargo FD, Gygi SP, Bartel DP. The impact of microRNAs on protein output. Nature 2008; 455: 64-71.
19. Eto K, Goto S, Nakashima W, Ura Y, Abe SI. Loss of programmed cell death 4 induces apoptosis by promoting the translation of procaspase-3 mRNA. Cell Death Differ 2012; 19: 573-81.
20. Jia L, Yu Z, Hui L, et al. Fas and FasL expression in the spinal cord following cord hemisection in the monkey. Neurochem Res 2011; 36: 419-25.
21. Povysheva TV, Mukhamedshina YO, Rizvanov AA, Chelyshev YA. PTEN expression in astrocytic processes after spinal cord injury. Mol Cell Neurosci 2018; 88: 231-9.
22. Redell JB, Zhao J, Dash PK. Altered expression of miRNA-21 and its targets in the hippocampus after traumatic brain injury. J Neurosci Res 2011; 89: 212-21.
23. Sun G, Lu Y, Li Y, et al. miR-19a protects cardiomyocytes from hypoxia/reoxygenation-induced apoptosis via PTEN/PI3K/p-Akt pathway. Biosci Rep 2017; 37: BSR20170899.
24. Liu XS, Chopp M, Wang XL, et al. MicroRNA-17-92 cluster mediates the proliferation and survival of neural progenitor cells after stroke. J Biol Chem 2013; 288: 12478-88.
25. Casha S, Yu WR, Fehlings MG. Oligodendroglial apoptosis occurs along degenerating axons and is associated with FAS and p75 expression following spinal cord injury in the rat. Neuroscience 2001; 103: 203-18.
26. Chli C, Benveniste EN. Fas ligand/Fas system in the brain: regulator of immune and apoptotic responses. Brain Res Rev 2004; 44: 65-81.
27. Demjen D, Klussmann S, Kleber S, et al. Neutralization of CD95 ligand promotes regeneration and functional recovery after spinal cord injury. Nat Med 2004; 10: 389-95.
28. Jiang XM, Yu XN, Liu TT, et al. microRNA-19a-3p promotes tumor metastasis and chemoresistance throughthe PTEN/Akt pathway in hepatocellular carcinoma. Biomed Pharmacother 2018; 105: 1147-54.
29. Wang Y, Zhao S, Zhu L, Zhang Q, Ren Y. MiR-19a negatively regulated the expression of PTEN and promoted the growth of ovarian cancer cells. Gene 2018; 670: 166-73.
30. Zhang X, Chen Y, Zhao P, Zang L, Zhang Z, Wang X. MicroRNA-19a functions asan oncogene by regulating PTEN/AKT/pAKT pathway in myeloma. Leuk Lymphoma 2017; 58: 932-40.
31. Liu C, Wu J, Xu K, et al. Neuroprotection by baicalein in ischemic brain injury involves PTEN/AKT pathway. J Neurochem 2010; 112: 1500-12.
32. Zhang QG, Wu DN, Han D, Zhang GY. Critical role of PTEN in the coupling between PI3K/Akt and JNK1/2 signaling in ischemic brain injury. FEBS Lett 2007; 581: 495-505.
33. Shi GD, Ouyang YP, Shi JG, Liu Y, Yuan W, Jia LS. PTEN deletion prevents ischemic brain injury by activating the mTOR signaling pathway. Biochem Biophys Res Commun 2011; 404: 941-5.
34. He Q, Cai L, Shuai L, et al. Ars2 is overexpressed in human cholangiocarcinomas and its depletion increases PTEN and PDCD4 by decreasing microRNA-21. Mol Carcinog 2013; 52: 286-96.
35. Asangani IA, Rasheed SA, Nikolova DA, et al. MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer. Oncogene 2008; 27: 2128-36.
36. Zhang H, Ozaki I, Mizuta T, et al. Involvement of programmed cell death 4 in transforming growth factor-beta1-induced apoptosis in human hepatocellular carcinoma. Oncogene 2006; 25: 6101-12.
37. Zhang G, Zha J, Liu J, Di J. Minocycline impedes mitochondrial-dependent cell death and stabilizes expression of hypoxia inducible factor-1alpha in spinal cord injury. Arch Med Sci 2019; 15: 475-83.
38. Eken MK, Ersoy GS, Kaygusuz EI, et al. Etanercept protects ovarian reserve against ischemia/reperfusion injury in a rat model. Arch Med Sci 2019; 15: 1104-12.