Original article:

Sirtuin 1 (SIRT1) and activated caspase 3 expression on rat spinal cord in acute phase after Sciatic nerve injury

Dwi Nur Ahsani[1], Rina Susilowati[2], Yustina Andwi Ari Sumiwi[2]

Abstract

Background: Increase of SIRT1 expression inhibit the increase of activated Caspase 3 expression in acute phase of traumatic brain injury (TBI). However, the SIRT1 expression on rat spinal cord in acute phase after peripheral nerve injury was unknown. ***Objective***: To reveal SIRT1and activated Caspase 3 expression on rat spinal cord in acute phase after sciatic nerve injury and to reveal the correlation between SIRT1 and activated Caspase 3 expression after sciatic nerve injury. ***Method:*** Thirty male Wistar rats aged 3 months were divided into sham operated and crush injury group. Termination was performing at days 3, 7 and 14. Lumbar spinal cord segments 4-5 (VTh12-VL1 high) embedded in paraffin blocks. Imunohistochemistry staining was performed using antibody of SIRT1 nuclear marker and activated Caspase 3. Observations were performed by two double blinded observers in 400x magnification. The data were analyzed using unpaired *t*-test and Pearson correlation. All data were processed using statistical analysis with confidence interval (CI) 95% and limit of significance (p-values)<0.05. ***Results:*** SIRT1 expression on the anterior horn was higher compared with control at days 7 and 14 ($p<0.05$), but no differences were found on posterior horn at all termination days($p>0.05$). Activated Caspase 3 expression on the anterior horn was higher at all termination days($p<0.05$) and at day 7 on posterior horn ($p<0.05$). Thereis a positive correlation between SIRT1 and activated Caspase 3 expression on anterior horn at day 7 ($p<0.05$). ***Conclusion:*** SIRT1 expression on the anterior horn was higher compared with the control at days 7 and 14. Activated Caspase 3 expression on the anterior horn was higher on all termination days and at day 7 on posterior horn. There is a positive correlation between SIRT1 and activated Caspase 3 expression on anterior horn on day 7.

Keywords: SIRT1; cells apoptosis; spinal cord; crush injury

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Introduction

Damage to peripheral nerves is caused by various etiologies. Two major etiology causes are injury and surgery. Injury contributes as much as 87% of peripheral nerve damage. Peripheral nerve injury can occur at various locations. Eleven percent of peripheral nerve injury occurs in the inferior extremities.[1] Peripheral nerve injury in the inferior extremities is important because it can cause an impairment to do mobilization.

Regeneration process will be induced after peripheral nerve injury. Regeneration process will facilitate a new axons bud formation in the distal part of lesion. A perfect regeneration process is characterized by the formation of new axons bud without the occurrence of neurological disorders. Interruption in regeneration process often occurs and induces regeneration disorders. These regeneration disorders are characterized by a slow growth of new axons as well as the emergence of variety neurological disorders.[2-4]

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One of the causes of neurological disorders after peripheral nerves injury are cells apoptosis on spinal cord. Cells apoptosis on the posterior horn of spinal cord would induce sensory neuron disturbances, and cells apoptosis on the anterior horn of spinal cord would induce motor neuron disorders.[3,5-7] Injury on sciatic nerve is often used to study the occurrence of cells apoptosis after peripheral nerve injury on the experimental animals. Scholtz *et al.* (2005) reported that sensory interneurons cells apoptosis on the posterior horn of rat spinal cord occurred on days7, 14, and 21 after sparing nerve injury on sciatic nerve. The peak phase of cell apoptosis occurred on day 7.[6] One of the parameters used to determine the occurrence of cell apoptosis was activation of Caspase 3 which was the effector of Caspase system.[8]
Sirtuin1 (SIRT1) is a histone deacetilase enzyme class III (HDAC III) that plays an important role in substrate deacetylation. SIRT1 substrates include histone and non-histone (transcription factors and co-enzyme) proteins. Research showed that non-histone protein deacetylation played an important role in neuroprotective mechanism. Deacetylation in various transcription factors will activate anti-apoptotic genes and inhibit pro-apoptotic genes.[9-11]
Pfister *et al.* (2008) reported that neuroprotective mechanism of SIRT1 was not influenced by its location in cell.[12] Cell apoptosis on rat central nervous system is regulated by the interactions between mitogen activating protein kinase/extracellular signal-regulated kinase pathways (MAPK / ERK) and Sirtuin1 (SIRT1) in the cytoplasm. Zhao *et al.* (2012) reported that there was an increase in activated Caspase 3 expression, SIRT1 expression, and MAPK expression in acute phase of TBI on in-vivo and in-vitro studies. Inhibition of SIRT1 pathway by administration of siRNA and SIRT1 inhibitors will increase the activated Caspase 3 expression. In contrast, inhibition of MAPK/ ERK pathway will decrease the activated Caspase 3 expression.[13]
Various studies have shown that administration of SIRT1 activator increases neuroprotective response to various cell death stimulation. Intra vitreal injection of SIRT1 activator in acute phase is able to inhibit retinal ganglion cells death in rat model experimental autoimmune encephalomyelitis (EAE).[14] Increase of SIRT1 expression in rat model EAE reduce paralysis incidence, inhibit inflammation, demyelination and prevent spinal cord cells apoptosis. Increase of SIRT1 expression can improve motor function, reduce brain atrophy, and increase neuronal cells survival in animal models of Huntington.[15] Increase of SIRT1 expression also increased the brain derived neurotrophic factor (BDNF) expression on spinal cord which is needed in peripheral nerves regeneration.[16]
Seeing the important role of SIRT1 in preventing cell death in acute phase in central nervous system, more studies are needed to investigate the role of SIRT1 in acute phase after peripheral nerve injury. This study will examine the role of SIRT1 on rat spinal cord in acute phase after sciatic nerve injury**.**

Materials and method

Sciatic nerve injury and termination

Thirty male Wistar rats aged 3 months and weighing 200-230 gr were divided into crush injury and sham operated group randomly. The rats from both groups were anesthetized using ketamine xylazin solution intramuscularly on the left thigh. After the rat slept, the posterior right thigh were cleaned and opened to find the sciatic nerve. Crush injury was performed at 0.5 cm proximal from the point entry of sciatic nerve to the muscle in the lower right leg (mid-thigh) by using a serrated clamps at 4 mm width with 5.24 kg grip strength (30 seconds for each side).
Irrigation using penicillin-streptomycin solution was conducted to prevent infection. Skin suturing was done aseptically. To prevent infection and reduce pain, the rats were given water containing antibiotics (amoxicillin) and analgesic (ibuprofen) ad-libitum for 3 days. The rats were placed in the different cages. Five rats each group was sacrificed by transcardial perfusion on days3, 7, and 14 by using 10% formalin in PBS to collect the spinal cord. The sample were embedded in paraffin blocks with transversal orientation.

Immunohistochemical staining and imaging

Three slices with 3 μm thickness and 100 μm distance were used for imunohistochemistry(IHC) staining for each antibody. Deparaffinization was performed using xylol and hydration by using alcohol. Tris EDTA (for anti-SIRT1 antibody) and PBS tween 0,1% (for anti-activated Caspase 3) were used as the washing solution. The slices was incubated for 15 minutes with 0,3% hydrogen peroxide in methanol, 20 minutes antigen retrieval (med-low) and 10 minutes background sniper. Primary antibody incubation at 4^0C overnight (1:400 anti-SIRT1 antibody nuclear marker ab110304 Abcam, 1:150 anti-activated Caspase 3 antibody ab13847 Abcam). Universal Trekki link was performed for 10 minutes and 10 minutes of avid in biotin-HRP. Incubation of DAB 1:100 for 23 minutes on anti-SIRT1 antibody, 1:300 for 2 minutes on anti-activated Caspase 3 antibody and 2 minutes counter stain with Hematoxylin.

Observation was perform in five visual fields on the anterior and three on the posterior horn (400x magnification using a light microscope attached with optilab).

Data analysis

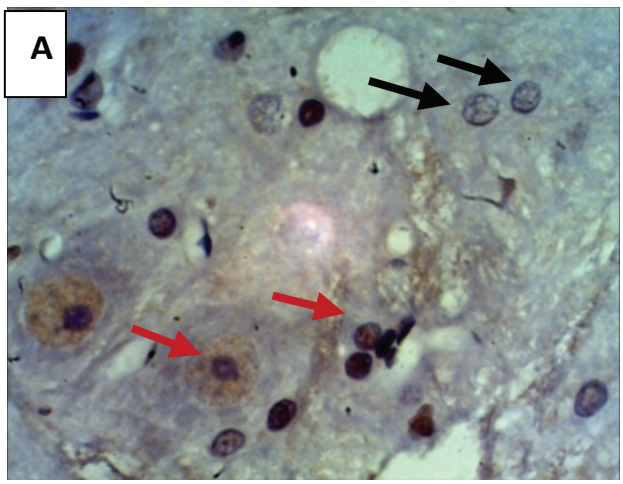
Mean percentage of the imunopositive cells on each group was obtained from two observers in each cornu. The data were analysed using normality test (Shapiro Wilk). Unpaired *t*-test was used to determine the significance of data, mean while, Pearson correlation was used to determine the correlation between SIRT1 and activated Caspase 3 expression. All data were processed with statistical analysis program with confidence intervals (CI) 95 % and p-value<0.05.

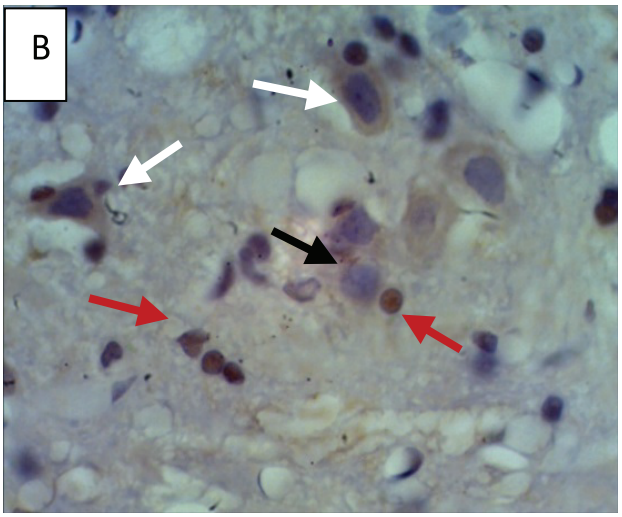
Ethical clearance: This study was approved by the Medical and Health Research Ethics Committee of Universitas Gadjah Mada, Yogyakarta, Indonesia .

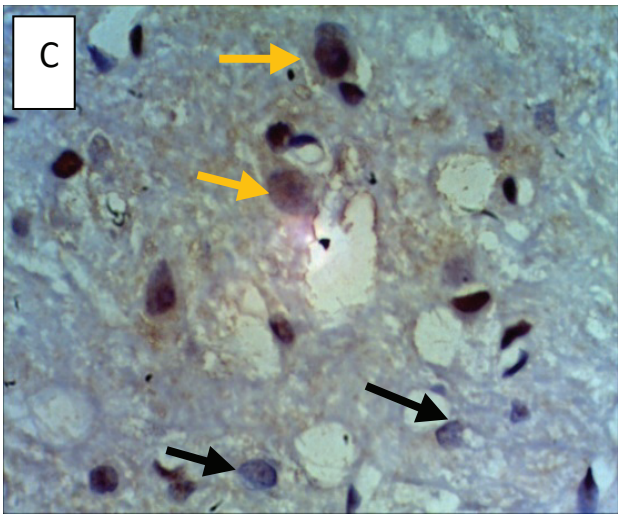
Results

SIRT1 and activated Caspase 3 expression could be found in both group. SIRT1 expression was predominant intranuclear, while cytoplasmic expression could be found in several large neurons. Activated Caspase 3 expression could be found in cytoplasm (Image1 and 2).

SIRT1 expression on anterior horn was higher in crush injury group at day7 and 14 (p=0.020 and p=0.024, Figure 1). Activated Caspase 3 expression in crush injury group was also higher on all termination day (p=0.046, p= 0.019,p=0.045, Figure 2).SIRT1 expression on posterior horn was not different from control (p>0.05, Figure 3). Activated Caspase 3 expression was higher at day 7 (p=0.032, Figure 4). Positive correlation between SIRT1and activated Caspase 3 expression occurred on day 7 (p=0.020, Table 1). No correlation was found between SIRT and activated Caspase 3 expression on the posterior horn on all termination days(p>0.05, Table 2).







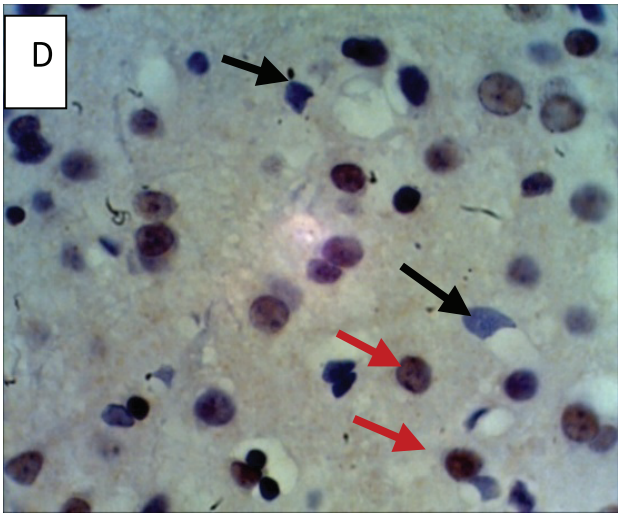
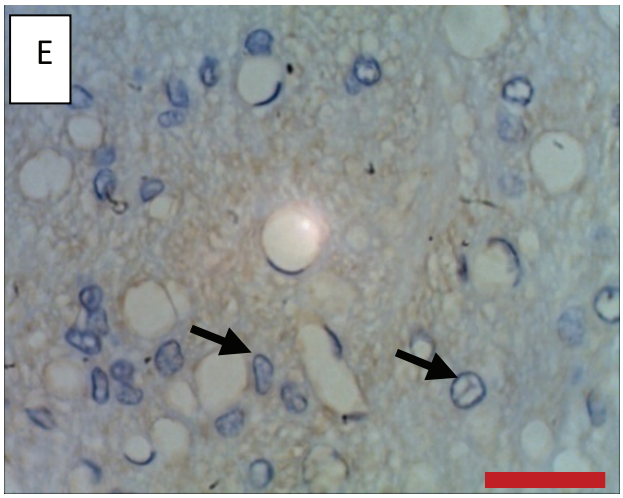
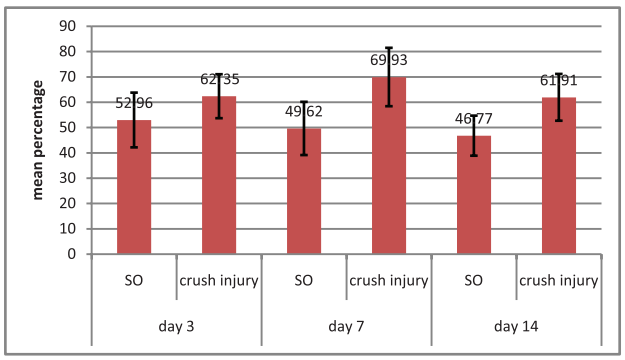


Image 1. Image of SIRT1 and activated Caspase 3 expression on anterior horn. A and B: SIRT1 expression on anterior horn. C: activated Caspase 3 expression on anterior horn. D: SIRT1 expression on rat hippocampus (control). E: negative control. Scale indicates 15 µm. (red arrows: intranuclear





*: p< 0,05, SO : Sham operated,

Figure 1. Unpaired t-test mean percentage of the SIRT1 imunopositive cells on anterior horn

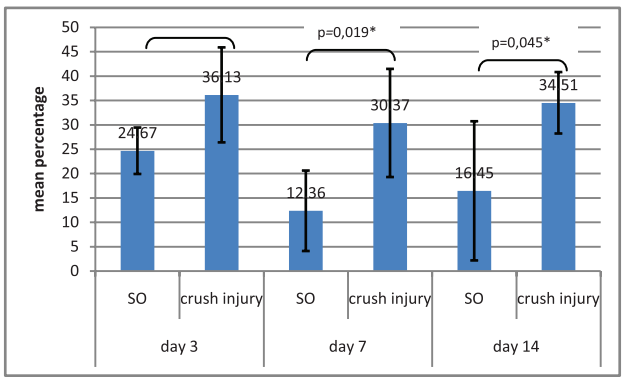
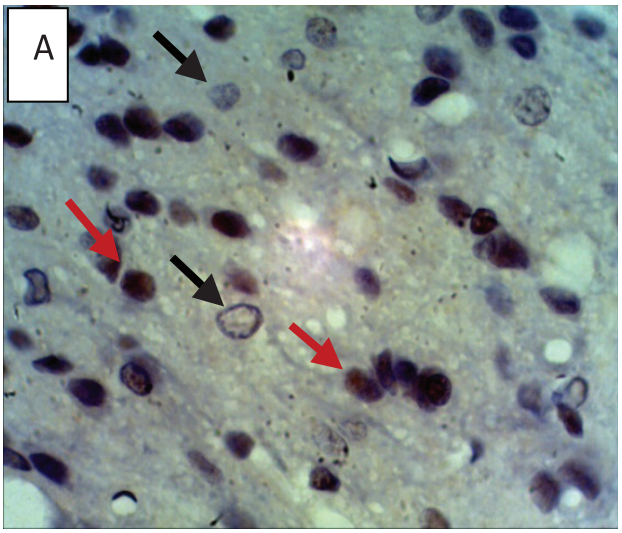


Figure 2. Unpaired t-test mean percentage of the activated Caspase 3 imunopositive cells on anterior horn

SIRT1 expression, white arrows : cytoplasmic SIRT1 expression, yellow arrow : caspase 3 expression, black arrows : imunonegative cells).

Table 1.Pearson correlation of SIRT1 and activated Caspase 3 expression on anterior horn

Termination	N	Correlation value	p
Day 3	10	0,331	0,350
Day 7	10	0,714	0,020*
Day 14	10	0,331	0,349



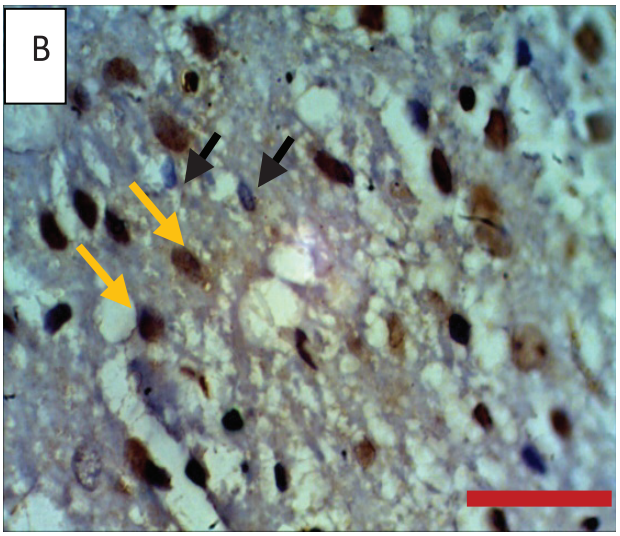


Image 2.SIRT1 and activated Caspase 3 expression on posterior horn. A: SIRT1 expression (red arrows). B: activated Caspase 3 expression (yellow arrows). Scale indicates 15 µm.

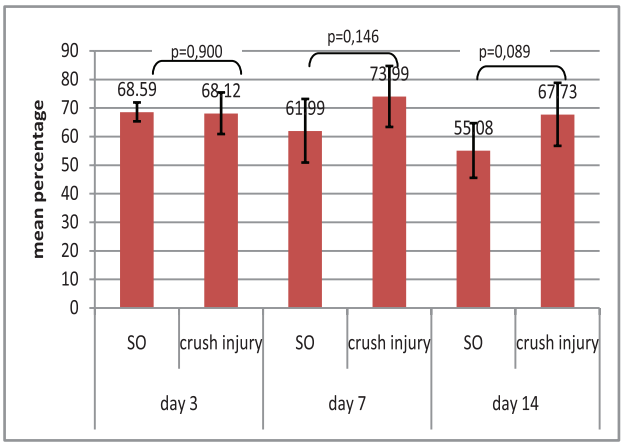


Figure 3. Unpaired t-test mean percentage of SIRT1 imunopositive cells on posterior horn

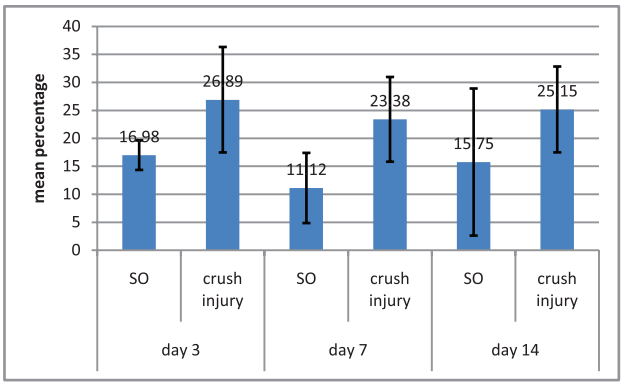


Figure 4. Unpaired t-test mean percentage of activated Caspase 3 imunopositive cells on posterior horn

Table 2.Pearson correlation test of SIRT1 and activated Caspase 3 expression on posterior horn

Termination	N	Correlation value	*p*
Day 3	10	0,527	0,118
Day 7	9	0,607	0,083
Day 14	10	0,027	0,940

Discussion

Crush injury on sciatic nerve in this study induce cells apoptosis. This is characterized by a higher activated Caspase 3 expression compared with the control. Neuronal cells apoptosis after peripheral nerve injury was caused by exitotoxicity and loss of neurotrophic factor.[17] Lost of contact between neuronal cells and target cells will induce neuronal cells apoptosis.[18]

A higher activated Caspase 3 expression in this research could be observed earlier on anterior horn than posterior horn.On the anterior horn, activated Caspase 3 expression could be detected from day3 until 14, while on the posterior horn on day 7. These results were consistent with Lawson and Lowrie (1998) who reported that motor neuron cell apoptosis occurred from day 3 to day 12 after crush injury.[19] Scholtz *et al.* (2005) reported that sensory interneurons cell apoptosis occurred from day7 to day 21 after SNI on sciatic nerve.[6]

The different occurrence of activated caspase 3 expression time between anterior and posterior horn was apparently caused by the difference in susceptibility to neurotrophic loss and also in anatomical location. Neurotrophic factors released by target cells are trophic dependent. Motor neurons on anterior horn require ciliary neurotrophic factor (CNTF), whereas sensory interneurons cells on dorsal horn require nerve growth factor (NGF) for survival. It seems that in this study, motor neuron cells on the anterior horn were more vulnerable to the loss of neurotrophic factor released by the target cells compared with the sensory interneurons cells on the posterior horn.[18] Anatomically, direct impact is felt by motor neurons on the anterior horn after crush injury while sensory interneuron cells on the posterior horn are protected indirectly by the presence of sensory neurons in dorsal root ganglia (DRG).[20]

Cells apoptosis after peripheral nerve injury was affected by the type, location, and duration of the injury. Crush injury will result in cells apoptosis at acute phase while ligation will result in cells apoptosis in chronic phase.[2,7] In line with that study, in this research, a higher activated Caspase 3 expression were found in acute phase.Cells apoptosis in the spinal cord would be higher if the distance between the location of injury and neuronal cells were closer and a longer duration of injury.[21] The long distance between location of crush injury and the sensory interneurons cells in this study resulted in an inadequate cells apoptosis induction. That's why a higher Caspase 3 expression on posterior horn can be found only on day7.

This study found that a higher activated Caspase 3 expression in the anterior horn will induce a higher SIRT1 expression in the same region. A higher activated Caspase 3 expression onday3 was also followed by a higher SIRT1 expression on day7. Similarly, a higher activated Caspase 3 expression on day-7 was also followed by a higher SIRT1 expression on day14. On the posterior horn, a higher activated Caspase 3 expression on day-7was not able to induce higher SIRT1 expression on day14. It seems that in order to be able to induce a higher SIRT1 expression, a longer duration of a higher activated Caspase 3 expression should be establish.

A positive correlation between SIRT1 and activated Caspase 3 expression on anterior horn of rat spinal cord on day 7 indicated that correlation occurred atan early detected time of a higher SIRT1 expression. The absence of correlation between SIRT1 and activated Caspase 3 expression on posterior horn showed that a short time increase of activated Caspase 3 expression was not able to induce a higher SIRT1 expression.

The use of nuclear marker SIRT1 antibody in this research shows that SIRT1 expression in the cytoplasmic was happens due to translocation form intranuclear. SIRT1 translocation can occur in both directions. SIRT1 translocation to the cytoplasmic induced by phosphoinositide 3-kinase (PI3K) and

insulin growth factor-1 (IGF-1) pathways.[22]

The intracellular location of SIRT1 expression affects SIRT1 neuroprotective mechanisms. The neuroprotective mechanisms of SIRT1 in intranuclear is performed through deacetylation of intranuclear substrate, while in cytoplasm occurs through deacetylation of cytoplasmic substrate and interactions with sitoplasmic pathways.[9,10,23] Zhao *et al*. (2012) stated that neuroprotective SIRT1 in cytoplasmic occurs due to interaction with MAPK pathways.[9] Results of this study showed that the dominant expression of SIRT1 in intranuclear indicated that the major neuroprotective mechanisms of SIRT1 on rat spinal cord in acute phase crush injury on sciatic nerve was through intranuclear deacetylation.

Conclusion

SIRT1 expression on the anterior horn was higher compared with the control at days 7 and 14. Activated Caspase 3 expression on the anterior horn was higher on all termination days and at day 7 on posterior horn. Thereis a positive correlation between SIRT1 and activated Caspase 3 expression on anterior horn on day7.

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Conflict of interest: There is no any conflict of interest for this publication.

Authors' Contributions: I also declare that all the authors mentioned in this manuscript have seen the final version of manuscript. I accept all the terms and conditions displayed in Bangladesh Journal of Medical Science. To the best of my knowledge. all authors have participated sufficiently in the conception and design of this work and the analysis of the data.

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