

## Original Article

# Early and late apoptosis protein expression (Bcl-2, BAX and p53) in traumatic brachial plexus injury

Heri Suroto<sup>1</sup>, Asyumaredha Asriel<sup>1</sup>, Brigita De Vega<sup>2</sup>, Steven K. Samijo<sup>3</sup><sup>1</sup>Department of Orthopaedic & Traumatology, Dr. Soetomo General Hospital/Universitas Airlangga, Surabaya, Indonesia;<sup>2</sup>Institute of Orthopaedic and Musculoskeletal Science, University College London, United Kingdom;<sup>3</sup>Department of Orthopaedic, Zuyderland Medisch Centrum, Heerlen, Netherlands**Abstract**

**Objectives:** This research aims to analyze the expression of pro-apoptotic proteins (Bax, p53) and anti-apoptotic protein (Bcl-2) in the nerve roots of the brachial plexus following traumatic brachial plexus injury (TBPI) in the early and late stage. **Methods:** A total of 30 biopsy samples were taken from the proximal stump of the postganglionic nerve roots of the TBPI patients' brachial plexus from January 2018 until September 2019. The samples were taken from patients within six months of trauma (early stage, group A) and more than six months following trauma (late stage, group B). Bcl-2, Bax, and p53 expressions in each group were measured and compared. **Results:** We found significant differences in the Bcl-2 ( $p=0.04$ ), Bax ( $p<0.0001$ ), p53 ( $p<0.0001$ ) expressions between group A and B. The Bcl-2/Bax expression ratio in group A and B was 2.26 and 0.22, respectively. Meanwhile, the Bcl-2/p53 expression ratio in group A and B was 1.64 and 0.23, respectively. **Conclusion:** Apoptosis is inhibited by Bcl-2 activities in the early stage following trauma. In the late stage, a significant decrease of Bcl-2 coupled with a substantial increase of Bax and p53 indicates a continuation of the apoptotic process.

**Keywords:** Anti-Apoptotic Protein, Brachial Plexus Neuropathies, Peripheral Nerve Injuries, Pro-Apoptotic Protein**Introduction**

The functional recovery of neurons after traumatic brachial plexus injury (TBPI) is poor and usually results in severely disabling motor and sensory deficit of the upper limb<sup>1</sup>. It causes a decrease in bodily functions, psychological disorders, and decreased social and economic ability of the patient. The poor functional result may at least be in part due to retrograde cell death among the injured neurons, which will restrict the capacity for peripheral nerve regeneration<sup>2</sup>. A proximal injury to the brachial plexus, however, results in a 30% loss of motor neurons. The corresponding time of cell death for motor neurons is weeks or months after the injury.

It has been shown that after injuries to the postganglionic plexus, the sensory neuron loss was reduced from about 50% to 25%, and the 30% motor neuron loss was almost totally eliminated<sup>3</sup>.

After an injury, there is an activation of both regenerative and cell death signaling processes in any type of neuron. It is the balance between those two processes that determine whether the neuronal cell could survive, regenerate, or die<sup>4</sup>. Neuronal cell death is regulated by an apoptosis pathway via pro-survival mediator protein Bcl-2 and the pro-apoptotic protein Bax, which interact at the mitochondrial level determining their membrane permeabilization<sup>5,6</sup>.

Their regulatory effect depends on its quantity of expression, although the Bcl-2/Bax ratio is considered as a key factor. The Bcl-2/Bax ratio increases when cell apoptosis is inhibited and, the other way around, the ratio will decrease when apoptosis is promoted<sup>7</sup>. It has been suggested that another protein, p53 expression, is important in neuronal injury. P53 is elevated in damaged neurons and acute models of injury; whereas, the absence of p53 has been shown to protect neurons from a wide variety of acute cytopathic insults<sup>8</sup>.

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**Table 1.** Demographic data of TBPI Patients.

		Group A ≤ 6 months	Group B > 6 Months
Sex	Male (patient/sample)	9 (45%) /14 (47%)	9 (45%) /12 (40%)
	Female (patient/sample)	1 (5%) /2 (7%)	1 (5%) /2 (7%)
Age	Mean (patient/sample)	28.00 ± 13.36 years/ 28.00 ± 13.44 years	27.10 ± 11.44 years/ 28.07 ± 12.10 years
Affected side	Left (patient/sample)	2 (10%)/3 (10%)	5 (25%)/5 (17%)
	Right (patient/sample)	8 (40%)/13 (43%)	5 (25%)/9 (30%)
Mode of Injury	MVA* (patient/sample)	10/16	10/14
BPI level	C5-7 (patient/sample)	4 (20%)/7 (23%)	3 (15%)/5 (17%)
	C5-T1 (patient/sample)	6 (30%)/9 (30%)	7 (35%)/9 (30%)
Time to surgery	Mean (patient/sample)	3.40 ± 2.07 months/ 3.44 ± 2.03 months	28.57 ± 37.62 months/ 28.00 ± 34.86 months
Surgical Procedure	Nerve Transfer/NT (patient/sample)	8 (40%)/13 (43%)	3 (15%)/5 (17%)
	FFMT* (patient/sample)	1 (5%)/1 (3%)	6 (30%)/7 (23%)
	Nerve grafting/NG (patient/sample)	1 (5%)/2 (7%)	1 (5%)/2 (7%)

Note: \*MVA: Motor vehicle accident, FFMT: Free functional muscle transfer.

**Table 2.** Comparison of Bcl-2, Bax, and p53 expression in biopsy samples of TBPI patients based on time to surgery.

Protein marker	Mean ± SD of positive cells*		p-value
	Group A(≤ 6 months)	Group B(> 6 months)	
Bcl-2	4.39 + 4.48	2.04 + 2.27	0.038
Bax	1.83 + 1.91	7.83 + 3.66	0.000
p53	2.68 + 3.20	7.57 + 5.08	0.004

Note: \*positive cells: motoneuron cell with brownish cytoplasm, indicating the cell is positively stained by the immunohistochemistry marker.

The apoptosis phenomenon of motor neurons in TBPI was well explored in animal models but was still lacking evidence in clinical practice<sup>5,7</sup>. Therefore, the objective of this study was to compare the expression of anti-apoptosis protein Bcl-2, pro-apoptosis Bax, and modulating protein p53 in motor neurons of the brachial plexus in traumatic BPI patients within 6 months after trauma and later.

## Methods

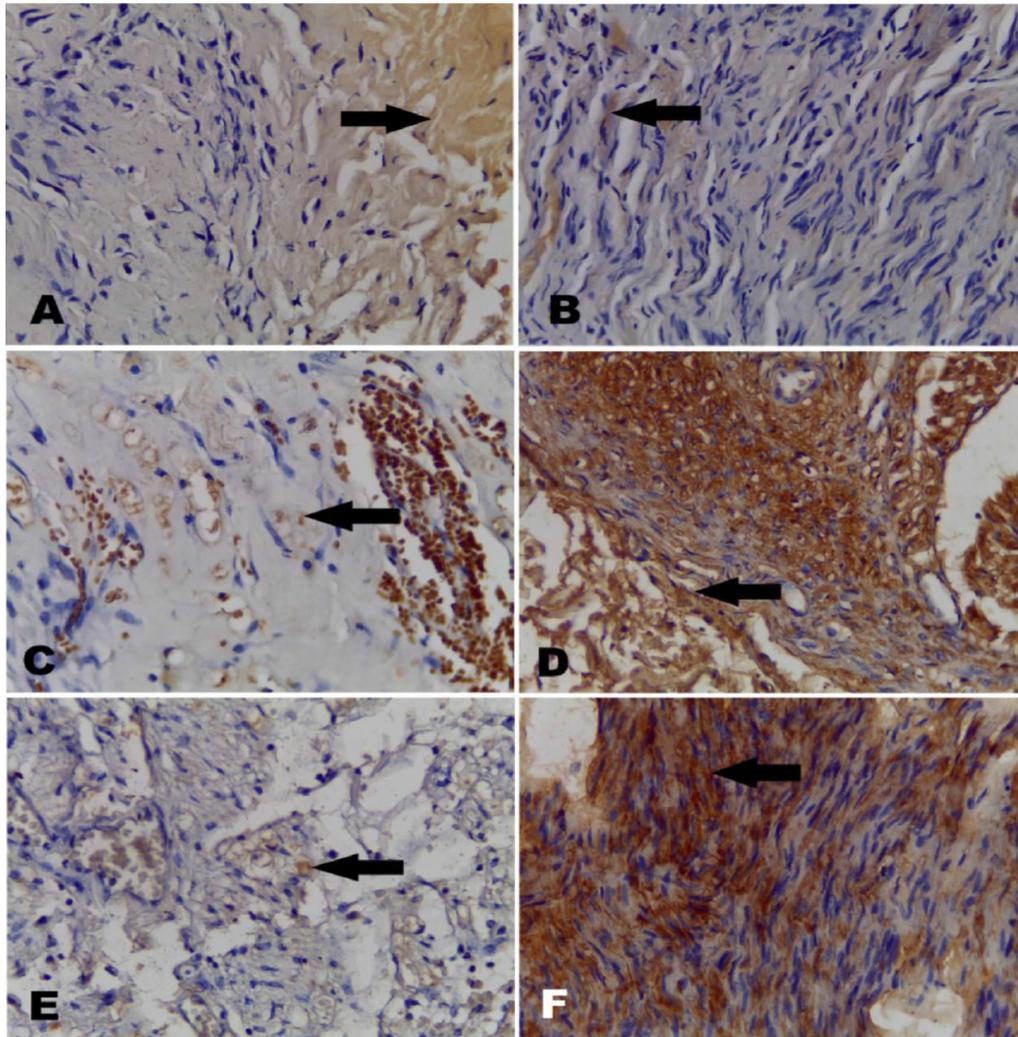
### Study design

This research was a cross-sectional study performed from January 2018 until September 2019 in TBPI, otherwise healthy patients who were older than 18 years. Consecutive samplings of postganglionic nerve roots of the brachial plexus were taken per operatively during surgery. Samples were taken from 2 groups of TBPI patients: group A in which subjects had surgery within 6 months (early stage), and group B in which subjects had surgery later than 6 months after brachial plexus injury (late stage). Patients with birth brachial plexus injury (BBPI) and multi-trauma were excluded.

### Data collection and interpretation

Biopsy samples of 0.5-1 cm were taken of the proximal stump of the affected nerve root that was freshened during surgical exploration. One until two samples per patient were taken. Samples were put on 10% of formaldehyde and then fixed in paraffin blocks. Then, we made 3 slides per biopsy sample for 3 immunohistochemical staining examinations (1 slide each). Examination of pro-apoptotic (Bax, p53) and anti-apoptotic (Bcl-2) marker were carried out by immunohistochemical staining using the Bax human monoclonal antibody (6F11) (Bioenzy®), p53 (SP5) human monoclonal antibody (Cell Marque™), and Bcl-2 (E17) human monoclonal antibody (Cell Marque™), respectively.

The outcome assessor was blinded regarding the biopsy samples' grouping. At 400x magnification under a microscope, the slides were divided into ten independent fields and inspected for immunohistochemical-stained positive cells (i.e., the motoneuron with brownish cytoplasm). We recorded the positive cells for each monoclonal antibody (Bcl-2, Bax, and p53) immunohistochemistry staining. All procedures were carried out at the department of anatomical



**Figure 1.** Microscopic findings of biopsy samples obtained from the proximal stump of the donor nerve root of the brachial plexus of TBPI patients (400x magnification). Arrows showed brownish cytoplasm stained with monoclonal antibodies (Bcl-2, Bax, and p53) immunohistochemistry. (A) Bcl-2 expression before 6 months, (B) Bcl-2 expression after 6 months, (C) Bax expression before 6 months, (D) Bax expression after 6 months, (E) p53 expression before 6 months, (F) p53 expression after 6 months (source: internal documentation).

pathology of our hospital. The study protocol was approved by the Health Research Ethics Committee of our hospital (certificate number 1548/KEPK/X/2019), and informed consent of the patient was obtained.

#### Data analysis

The positive cells data were presented in mean and standard deviation (mean  $\pm$  SD), then were analyzed with SPSS software (IBM SPSS Statistics Version 26 Copyright IBM Corporation and its licensors 1989, 2019). A normality test was conducted using Shapiro-Wilk test. When an abnormal distribution was encountered, a non-parametric Mann Whitney U test was conducted to analyze the data. Otherwise, we used a parametric test (independent t-test).

#### Results

The demographic of the patients included in this study is presented in Table 1. In total, 20 TBPI patients were included (group A: 10 patients, 16 samples; Group B: 10 patients, 16 samples), with equal male/female distribution (9 male and 1 female subjects in each group). All patients had TBPI due to motor-vehicle accidents. The average time to surgery in group A was  $3.4 \pm 2.07$  months and in group B was  $28.57 \pm 37.62$  months.

The expression of Bcl-2, Bax, and p53 in each group was observed and recorded. The Shapiro-Wilk test for normality showed that our data was not normally distributed, therefore we used non-parametric Mann Whitney U test to compare

the data (see Table 2). Significant differences were found in the expression of the observed markers between the 2 groups. Bcl-2 was expressed higher in group A (early period of trauma), while Bax and p53 were expressed higher in group B (late period of trauma). The Bcl-2/Bax expression ratio in group A was 2.26, while in group B was 0.22. The Bcl-2/p53 expression ratio in group A was 1.64, while in group B was 0.23. Figure 1 shows the microscopic findings of the biopsy samples.

## Discussion

Up until now, there is no explicit agreement on the ideal timing of surgical intervention for BPI. Although a systematic review comprising 43 studies suggested that the optimal time to surgery is shorter than 6 months<sup>9</sup>, there is no clear explanation regarding what occurs in the proximal stump of the postganglionic nerve roots before and after 6 months following injury. Previous literature has identified that there is a significantly higher expression of apoptotic proteins (caspase-3, caspase-8, and caspase-9) in the proximal stump biopsy of BPI patients who were operated on in the late stage (more than 6 months) than in the early stage (less than 6 months)<sup>10</sup>. However, a more comprehensive understanding of BPI pathomechanism (including other apoptotic and anti-apoptotic proteins) is essential to develop better management of BPI and other peripheral nerve injuries.

This study showed that in the early period after trauma, the Bcl-2 expression in motor neuron cells was higher, while Bax and p53 expression were lower. Commencing from 6 months after trauma, Bcl-2 expression was expressed lower, while Bax and p53 expression were higher. These findings indicate that the apoptosis activity in motor neurons of the brachial plexus is more prominent after 6 months of injury, and no counter mechanisms are active. Our findings are in agreement with the literature below.

Tian et al., using TUNEL and flow cytometry, found that the percentage of apoptotic muscle cells rose significantly in skeletal muscle atrophy. Early-stage morphological changes in apoptotic cells, such as chromosome aggregation, expansion of the core tank, and contraction of the nucleus, could be seen under the electron microscope. The Fas, FADD, and Caspase genes were expressed very high, and the Bcl-2 gene was expressed low, indicating an apoptosis process. However, changes in expression in the p53 and c-myc genes were unclear. Northern-blot results show that the Fas gene mRNA increase, the Bcl-2 gene decrease significantly in muscle atrophy caused by brachial plexus injury<sup>5</sup>.

Furthermore, another study conducted by Wang et al. showed that the Bcl-2 expression in motor neurons following nerve injury was decreased in its association with the death of motor neurons. This study also found that the reduction of Bcl-2 expression was correlated with two factors: the distance of the injury site to the nucleus and the type of injury. The nerve injuries that occurred in close proximity with the motor neuron nucleus experienced a significantly more prominent

Bcl-2 reduction compared to those occurring remote from the motor neuron nucleus. Whereas, the crush type nerve injury caused a more significant amount of Bcl-2 expression compared to nerve transection<sup>11</sup>.

In our study, the Bcl-2/Bax ratio was found to be higher early after trauma, compared to those later than 6 months. This was due to a high expression of Bcl2, also indicating low activity of apoptosis. Bcl-2 is an anti-apoptotic protein that protects cells from apoptosis by regulating the function of the mitochondrial membrane. Bcl-2-related X protein (Bax) encodes a pro-apoptotic protein that is responsible for inducing cell death. Bax excess causes cell death, while excessive Bcl-2 inhibits programmed cell death (apoptosis)<sup>5</sup>. Therefore, the Bcl-2/Bax ratio in cells is an essential determinant of apoptosis, and a decrease in the Bcl-2/Bax ratio is a factor in predisposing cells to apoptosis.

We found an increase in p53 expression on a later stage. Mitra and Dash (2018) claimed that to induce an apoptosis process, a large amount of modulator protein was needed, i.e. p53<sup>12</sup>. Following cell injuries, a substantial amount of p53 was produced. Since the member of Bcl-2 family proteins such as Bax is the product of p53, its expression was also elevated. This led to an increased proportion of Bax/Bcl-2 expression and eventually induced an apoptotic process<sup>12,13</sup>.

Moreover, Zhou et al. also mentioned that p53 played a vital role in inducing apoptosis. A large amount of p53 would be able to trigger the production of pro-apoptotic Bax protein and would reduce the Bcl-2 protein<sup>14</sup>. Our present study, supported by the aforementioned studies, showed that the p53 level was directly proportional to the Bax protein level and inversely proportional to the Bcl-2 protein. It was expected that there would be differences in the results of the handling of patients with brachial plexus injuries performed before six months because at this stage the process of apoptosis had not occurred much as a result of the high ratio of Bcl-2/Bax<sup>15,16</sup>.

By quantifying the anti- and pro-apoptotic protein expressions based on the time-to-surgery timeline, this study also sheds light on the rationale for the future development of nerve repair and nerve regeneration strategies. Various microsurgery options exist to treat BPI, namely direct end-to-end nerve repair (in a tension-free, minimal gap, clean-cut injury), nerve grafting, nerve transfer, and tendon/muscle transfer. Although direct end-to-end nerve repair is the gold standard, it is often impossible to perform, especially in delayed presentation, contusion/traction injury, or large gap defects. Thus, nerve grafting is considered the gold standard in these conditions<sup>17,18</sup>. Nevertheless, the viability of the injured nerve's proximal stump is crucial to determine the success of nerve grafting. Thus, by understanding the molecular processes that occur in the proximal stump, the future management of BPI can be directed at modulating the apoptotic and anti-apoptotic proteins so that neuroregeneration efforts can be achieved.

For instance, Liu et al. investigated the effect of adipose-derived stem cells (ADSC)-exosome on peripheral nerve injury repair. They found that the ADSC-exosome upregulated Bcl-2

mRNA expression and downregulated Bax mRNA expression, thus reducing Schwann cells apoptosis<sup>19</sup>. Likewise, Cong et al. also demonstrated that the administration of extracellular vesicles from skin precursor-Schwann cells showed neuron growth improvement through its similar effect in the modulation of Bcl-2 and Bax protein expression<sup>20</sup>. As the development of regenerative medicine nowadays is directed towards these biological agents, our present study may provide an indication for the administration of the novel therapy to augment the management of late-presenting BPI (more than 6 months following injury) or other peripheral nerve injuries where the Bcl-2/bax ratio is low.

In conclusion, our study shows valuable evidence that there are significant differences in the expression of Bcl-2, Bax, and p53 in motor neuron cells in patients with postganglionic type brachial plexus injuries before and at least 6 months after injury. Our present study supports the argument of specifying the 6-month cut-off point for TBPI surgical intervention choice as suggested by previous literature from its molecular standpoint, while also may give an indication for future therapeutic agents. The process of apoptosis is directly induced shortly after the traumatic event but is inhibited by anti-apoptosis (Bcl-2) mechanisms in the early stage. However, at 6 months following trauma, the pro-apoptotic proteins (Bax and p53) express themselves to trigger apoptosis (Bax and p53).

#### Ethical approval

*This study was reviewed and approved by the Ethics Committee of the Dr. Soetomo Hospital with reference number: 1399/KEPK/VIII/2019.*

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