Correlation of expression levels of caspase-3 and Bcl-2 in alveolar lavage fluid in neonatal respiratory distress syndrome and prognosis

YONGMEI LI[1], LU LIN[2] and QINGXI WANG[3]

Departments of [1]Pediatrics and [2]Neonatology, People's Hospital of Yucheng City, Yucheng, Shandong 251200; [3]Clinical Laboratory, Jinan Central Hospital, Jinan, Shandong 250013, P.R. China

Received June 30, 2017; Accepted October 17, 2017

DOI: 10.3892/etm.2018.5755

Abstract. This study was designed to investigate the correlation between expression levels of cysteine aspartic protease-3 (caspase-3) and B-cell lymphoma gene-2 (Bcl-2) proteins in alveolar lavage fluid and the prognosis of infants with neonatal respiratory distress syndrome (RDS). A total of 150 infants with neonatal RDS undergoing alveolar lavages were divided into four groups: RDS1 (group A, n=42), RDS2 (group B, n=38), RDS3 (group C, n=38) and RDS4 (group D, n=32) according to their thoracic X-ray film grading. The oxygen uptake score, oxygenation saturation, mean airway pressure and expression levels of caspase-3 and Bcl-2 in alveolar lavage fluid of the infants in the four groups were measured and compared. Our results showed higher grading by thoracic X-rays in patients with increased oxygen uptake score, oxygenation index, mean airway pressure, caspase-3 expression level, hospital stay, complications and death rates in all groups; however, the expression levels of Bcl-2 were decreased in those cases, and the differences had statistical significance among the four groups ($P<0.05$). Analyses for correlation showed a caspase-3 positive area that was positively correlated with oxygen uptake score, oxygenation index and mean airway pressure ($P<0.05$); and a Bcl-2 expression level that was negatively correlated with oxygen uptake score, oxygenation index and mean airway pressure ($P<0.05$). Based on our findings, the severity of neonatal RDS is positively correlated with the concentration of caspase-3 in alveolar lavage fluid, and negatively correlated with the expression level of Bcl-2.

Introduction

The mechanism of neonatal respiratory distress syndrome (RDS) is still under study, but some studies have suggested that oxidation/anti-oxidation disorders and inflammatory responses may play essential roles in the pathogenesis (1). However, new developments have indicated apoptosis as being another player. Cell apoptosis is a cellular process of programmed cell death under the influence of several gene products, and it is a recurring process necessary to maintain a healthy internal environment. Events leading to apoptosis disturbance may cause abnormal body development, deformation and even death, and apoptosis has been associated with a variety of diseases, such as endocrine disorders and tumors (2,3). It has been confirmed that B-cell lymphoma gene-2 (Bcl-2) and cysteine aspartic protease-3 (caspase-3) activate apoptosis cascades and are associated with the development of neonatal RDS (4). In this study, the expression levels of caspase-3 and Bcl-2 in alveolar lavage fluid of patients with neonatal RDS and different thoracic X-ray grades were compared, so as to investigate the correlation between the expression levels of caspase-3 and Bcl-2 and the prognosis of neonatal RDS.

Materials and methods

General material. In total, 150 infants with neonatal RDS who underwent alveolar lavage therapy in People's Hospital of Yucheng City from December, 2015 to October, 2016 were enrolled in the study. The diagnostic criteria of RDS in the fourth edition of 'Practical Neonatology' were adopted; progressive dyspnea occurred within 4 h after birth and was accompanied by expiratory grunts, results of thoracic X-ray examination included RDS3 or RDS4, and blood gas analysis showed PaO_2 <50 mmHg (6.6 kPa) or $PaCO_2$ >60 mmHg (7.8 kPa).

Inclusion criteria: i) The RDS occurred within 4 h of birth; ii) the gestational age was not more than 37 weeks; iii) the birth weight was less than 2.5 kg; iv) guardians of infants agreed to cooperate with the study and signed the informed consent.

Exclusion criteria: i) Patients diagnosed with infection in prenatal diagnosis; ii) infants with shock or severe asphyxia; iii) patients with lung or cardiac dysplasia; iv) patients with severe genetic diseases; v) infants with suspected chromosomal disease. The study was appoved by the Ethics Committee of People's Hospital of Yucheng City.

Correspondence to: Dr Lu Lin, Department of Neonatology, People's Hospital of Yucheng City, 753 Kaituo Road, Yucheng, Shandong 251200, P.R. China
E-mail: d7260910@163.com

Key words: respiratory distress syndrome, alveolar lavage fluid, caspase-3, B-cell lymphoma gene-2

Grouping of subjects. Hundred and fifty patients with neonatal RDS receiving alveolar lavage therapy were divided into four groups: RDS1 (group A, n=42), RDS2 (group B, n=38), RDS3 (group C, n=38) and RDS4 (group D, n=32) according to thoracic X-ray film grading. The RDS X-ray grading comprised four classifications: In RDS1, the transparency of the whole lung was reduced or a diffuse net and particle shadow appeared on both lungs, but the contours of the heart shadow could be clearly observed. In RDS2, the transparency of the whole lung was reduced, a diffuse particle shadow appeared, patchy high density shadow spots could be observed in partial pulmonary fields, the lung markings could not be identified, and significant air bronchogram was present. In RDS3, the transparency of the whole lung was reduced significantly, a large-particle shadow covered the lungs, air bronchogram was present, all lung markings disappeared, and the heart and diaphragmatic surfaces were unclear. In RDS4, a compact shadow was uniform across the pulmonary field, air bronchogram was partially clear or unclear, heart and diaphragmatic surfaces could not be identified, and white lung syndrome occurred.

Research methods

Bronchoalveolar lavage therapy. In this study, 150 cases of neonatal RDS underwent bronchoalveolar lavage with a bronchofiberscope. Briefly, all subjects received conventional sedation. A tracheal catheter was used to drop 37˚C sterile saline (0.5 ml/kg each time) into the bronchial tubes. Oxygen was monitored and supplied three times using a resuscitator. A suction tube was inserted into the tracheal catheter or lifted up slightly when faced with resistance. Aspiration was done under negative pressure of less than 6 kPa, the catheter was gradually withdrawn within 0.5 min. The procedures were performed three times on both sides of the bronchi. The alveolar lavage fluid retrieved was centrifuged at 3,200 x g for 10 min and the supernatant was collected.

Monitoring and recording of clinical variables. Conventional blood pressure, heart rate and respiratory monitoring were performed for all infants. After the procedures, the oxygen uptake score, oxygenation index and mean airway pressure were analyzed.

The Apgar score 1 min after birth was used to evaluate the neonate's condition. The hospital stays, complication and death rates of all patients were recorded, and the expression levels of caspase-3 and Bcl-2 in the alveolar lavage fluid were determined.

Detection of the protein expression level of caspase-3 in bronchoalveolar lavage fluid via enzyme-linked immunosorbent assay (ELISA). Standard ELISA experiments were conducted to determine the bronchoalveolar lavage fluid levels of caspase-3. A coating diluent of antibody was added to each sample at appropriate concentrations and incubated at 37˚C for 4 h. Sealing of enzyme-labeled reaction wells was achieved using 5% calf serum at 37˚C for 40 min. Next, the samples to be tested were added and diluted to 1:100; enzyme-labeled antibodies were added and diluted to 1:40. Finally, the substrate solution TMB-hydrogen peroxide urea solution was added in the dark at 37˚C for 5 min, followed by termination of the reaction and color development; and the wavelength at 450 nm was detected.

Table I. PCR primers.

Gene	Primer sequences	Primer length (bp)
β-actin	F: 5'-GCCAACACAGTGCTGTCTG-3'	
	R: 5'-CACATCTGCTGGAAGGTGG-3'	185
Bcl-2	F: 5'-TTCTTTGAGTTCGGTGGGGTC-3'	
	R: 5'-TGCATATTTGTTTGGGGCAGG-3'	96

PCR, polymerase chain reaction; F, forward; R, reverse.

Detection of Bcl-2 gene expression levels in bronchoalveolar lavage fluid via polymerase chain reaction (PCR). An RNA extraction kit was used to extract the total RNA in the samples in strict accordance with the manufacturer's instructions. After gel electrophoresis, the concentration of RNA was determined using a photometer (Bio-Rad Laboratories, Hercules, CA, USA). For annealing of primers, 2 μg RNA samples and 4 μl of OligoPrimer were added into 18 μl non-enzyme water at 70˚C for 10 min. Then 8 μl 5X buffer, 2 μl dNTP mixture, 1 μl RNase inhibitor and RNaseM-MLV12Ul were added and supplemented with non-enzyme water to 40 μl. The reactions were incubated at 42˚C for 60 min and then at 70˚C for 15 min. After the reaction, the samples were stored in a refrigerator. At a later time, PCR quantitative detections were carried out under standard conditions using 40 cycles of 95˚C for 30 sec, and 60˚C for 30 sec, in a thermocycler. See Table I for primer sequences.

Statistical analysis. SPSS 20.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis in this study. Analysis of variance was used for measurement data. The results are presented as mean ± standard deviation. The statistical methods used in the study included t-test, Chi-square test and Pearson's correlation analysis. A $P<0.05$ suggested that a given difference was statistically significant.

Results

Neonatal Apgar scores of child patients in the four groups. General survey results showed that there were no significant differences in terms of gestational age, body weight and time from birth to onset of symptoms among the infants in the four groups ($P>0.05$). The Apgar scores at 1 min after birth in groups A-D were low, and differed in a statistically significant manner among the four groups, with scores decreasing with each increase in severity evidenced by the X-ray classification group ($P<0.05$) (Table II).

Comparisons of oxygen uptake score, oxygenation index and mean airway pressure among the four groups. The patients with higher thoracic X-ray grading classification (from groups A to D) with RDS had the oxygen uptake scores, oxygenation indexes and mean airway pressures higher with each step in the severity classification ladder, and there were statistically significant differences among the four groups ($P<0.05$) (Table III and Figs. 1-3).

Table II. Neonatal Apgar scores of child patients in the four groups.

Group	Gestational age (weeks)	Body weight (kg)	Apgar score (1 min after birth)	Time from birth to onset (hours)
Group A (n=42)	33.75±1.53	2.12±0.43	6.91±0.86	3.57±0.11
Group B (n=38)	32.78±2.31	2.05±0.39	6.50±0.92	3.09±0.51
Group C (n=38)	33.09±1.67	2.10±0.46	6.23±0.41	3.24±0.33
Group D (n=32)	33.41±1.61	2.01±0.47	5.78±0.30	3.15±0.40
χ^2	0.05	0.29	15.741	2.374
P-value	0.12	0.35	0.01	0.66

Table III. Comparisons of oxygen uptake scores, oxygenation indexes and mean airway pressures among the four groups.

Group	Oxygen uptake score	Oxygenation index	Mean airway pressure
Group A (n=42)	0.39±0.07	11.52±2.90	8.90±1.77
Group B (n=38)	0.47±0.12	15.26±3.12	10.37±2.12
Group C (n=38)	0.54±0.11	22.50±4.08	13.04±2.01
Group D (n=32)	0.67±0.10	32.83±5.05	14.67±1.17
χ^2	7.845	11.303	7.920
P-value	<0.001	0.002	0.002

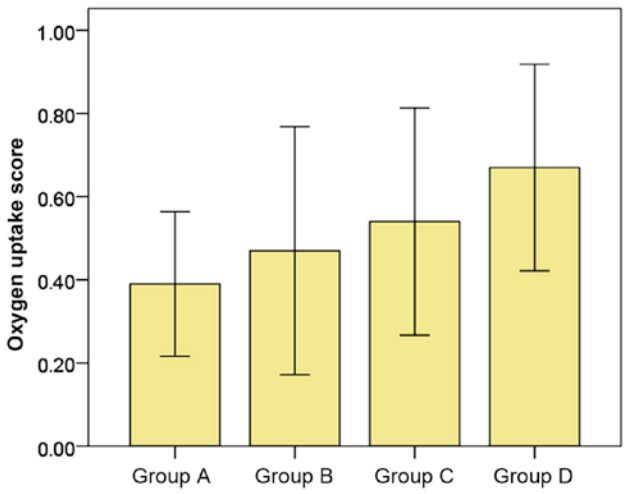

Figure 1. Bar chart of oxygen uptake score in the four groups.

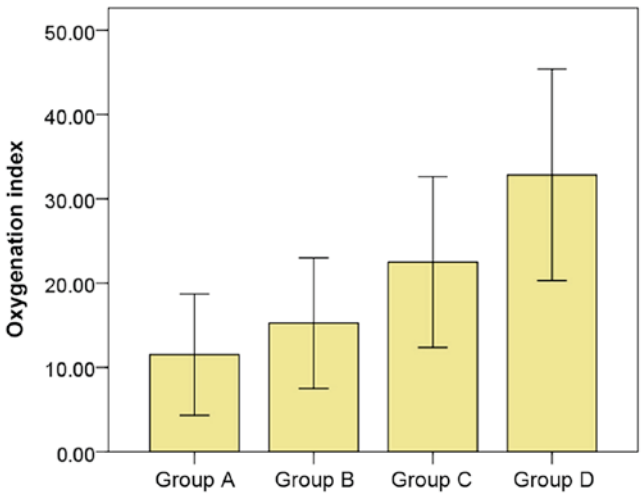

Figure 2. Bar chart of oxygenation index in the four groups.

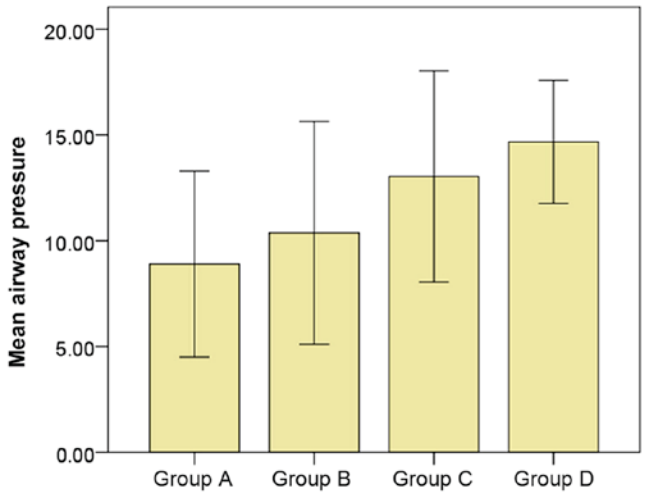

Figure 3. Bar chart of mean airway pressure in the four groups.

Comparisons of caspase-3 and Bcl-2 expression levels in alveolar lavage fluid among the four groups. The average caspase-3 expression levels in alveolar lavage fluid increased from group A to D. On the contrary, the average Bcl-2 expression levels decreased from group A to D; and there were statistically significant differences among the four groups (P<0.05) (Table IV).

Comparisons of hospital stay lengths, complication and death rates among the four groups. The hospital stay lengths, and complication and death rates got significantly worse in a sequential manner from groups A to D (P<0.05) (Table V).

Analysis of correlations of caspase-3 and Bcl-2 expression levels in alveolar lavage fluid with oxygen uptake score, oxygenation index and mean airway pressure. The correlations of alveolar lavage fluid expression levels of caspase-3 and Bcl-2 with oxygen uptake score, oxygenation index and mean airway pressure, in infants with RDS, were analyzed using Pearson's correlation analysis. The results showed a caspase-3

Table IV. Comparisons of caspase-3 and Bcl-2 expression levels in alveolar lavage fluid among the four groups.

Group	Caspase-3 expression level (ELISA, ng/l)	Bcl-2 expression level (PCR)
Group A (n=42)	38.20±5.06	4.85±0.45
Group B (n=38)	41.78±6.53	4.05±0.22
Group C (n=38)	46.39±6.21	3.27±0.08
Group D (n=32)	51.07±9.46	3.01±0.09
χ^2	8.873	9.762
P-value	0.001	<0.001

ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

Table V. Comparisons of hospital stay lengths, and complications and death rates among the four groups.

Group	Hospital stays (day)	Complications (n)	Death rate (n, %)
Group A (n=42)	13.37±3.35	3.14±0.94	2/42 (4.76)
Group B (n=38)	18.32±4.31	4.17±0.61	4/38 (10.53)
Group C (n=38)	20.14±3.09	6.34±1.21	5/38 (13.16)
Group D (n=32)	24.65±6.37	9.55±1.64	8/32 (25.00)
χ^2	8.546	7.322	7.397
P-value	0.002	0.012	0.003

positive area positively correlated with oxygen uptake score, oxygenation index and mean airway pressure (P<0.05); and a Bcl-2 expression level that was negatively correlated with oxygen uptake score, oxygenation index and mean airway pressure (P<0.05). See Table VI for values.

Table VI. Analysis of correlations of caspase-3 and Bcl-2 expression levels with oxygen uptake score, oxygenation index and mean airway pressure.

Index	Oxygen uptake score	Oxygenation index	Mean airway pressure
Caspase-3 positive area	0.039	0.022	0.015
Bcl-2 expression level	-0.003	-0.021	-0.012

Discussion

Neonatal RDS refers to a condition with respiratory failure and progressive respiratory distress shortly after the birth of an infant due to the lack of pulmonary surfactant and end-expiratory alveolar collapse, and in which an initial symptom is respiratory acidosis. In case of deterioration, the condition develops into metabolic acidosis, greatly affecting the life quality and survival rate of the newborn (5,6). The main clinical manifestations include cyanosis, polypnea, respiratory moaning inspiratory three depressions sign, and air bronchogram and ground-glass changes on chest X-rays. It is accepted that neonatal deaths due to neonatal RDS account for ~30% of the total neonatal deaths, and neonatal RDS is one of the major diseases leading to serious sequelae and death of the newborn. The treatment of neonatal RDS aims at reducing mortality rates, improve the prognosis and decrease the incidence of complications.

The development of multiple complications is closely related to degree to which apoptosis is stimulated. The process of apoptosis is very complex and usually influenced by the caspase and the Bcl-2 family of factors (7,8). Caspase-3 plays an important role in the protease cascade cleavage, which can hydrolyze apoptotic proteins in cells, promoting apoptosis of such cells. Other pathways like the mitochondrial pathway are also important; when an apoptosis factor stimulates cells, mitochondria can produce apoptosis factors, inducing cell death (9-11). In the relationship between caspase-3 and neonatal RDS, caspase-3 is involved in the pathogenesis of neonatal RDS in three ways. First, it affects the activity and functions of major regulatory proteins effectively producing phagocytosis. Second, it directly damages lung tissue cells. Caspase-3 can cleave Lamin A, thus causing chromatin aggregation, nuclear lamina collapse and changes in the nucleus. Third and finally, caspase-3 can reduce the activity of DNase inhibitors and alter the homeostatic level. It is known that the expression of caspase-3 protease rises in the initial stages of acute necrotizing pancreatitis-associated lung injury in rats, and reaches a peak after 48 h (12). In this study, the expression levels of caspase-3 in alveolar lavage fluid increased with the severity of the condition in the patients (from patients in groups A to D), and there were statistically significant differences among the four groups (P<0.05). Furthermore, Pearson's analysis showed a caspase-3 positive area that was positively correlated with oxygen uptake score, oxygenation index and mean airway pressure (P<0.05). These findings are consistent with the degree of pathological damage of the lung tissues in neonatal RDS patients, suggesting that the increased protein expression of caspase-3 has a certain impact on lung injury.

The Bcl-2 family of proteins is prevalent in humans and animals, where its main role is to inhibit apoptosis and promote proliferation. The Bcl-2 gene inhibits apoptosis in three ways (13-15): First, through its action as an antioxidant; second, through its inhibition of the production of endoplasmic reticulum calcium ions; and finally, through its interaction with other apoptosis factors like Bax. Activated Bcl-2 can promote the migration and proliferation of endothelial cells and inhibit apoptosis (16-18). It is generally believed that the pathogenesis of neonatal RDS is due to the large-area of damage to thealveolar-capillary membrane, which in turn causes atelectasis and pulmonary edema. During the pathological process, pulmonary vascular and alveolar epithelial endothelial cells are progressively induced into apoptosis, and this apoptosis mode may be related to the mediation by inflammatory factors. The Bcl-2 gene inhibits apoptosis of cells, antagonizes caspase-3, makes the cells resistant to apoptosis-stimulating factors, inhibits the decomposition of proteins in cells and prolongs the cell survival

cycle. Even though the link between apoptosis and inflammation is still being actively studied, both processes are closely related to the expression of the Bcl-2 protein. In this study, the average expression levels of Bcl-2 in alveolar lavage fluid decreased sequentially from groups with less severe disease to those with more severe conditions (from group A to D), and there were statistically significant differences among the four groups ($P<0.05$). Moreover, the expression level of Bcl-2 was negatively correlated with oxygen uptake score, oxygenation index and mean airway pressure ($P<0.05$). The above results suggest that apoptosis is very common in neonatal RDS and the inhibition and low expression levels of Bcl-2 may be the key in cases of worsening disease (19,20).

In conclusion, the severity of neonatal RDS is positively correlated with the caspase-3 expression level, but negatively correlated with the Bcl-2 expression in alveolar lavage fluid of child patients.

References

1. Frutos-Vivar F, Nin N and Esteban A: Epidemiology of acute lung injury and acute respiratory distress syndrome. Curr Opin Crit Care 10: 1-6, 2004.
2. Arakaki R, Yamada A, Kudo Y, Hayashi Y and Ishimaru N: Mechanism of activation-induced cell death of T cells and regulation of FasL expression. Crit Rev Immunol 34: 301-314, 2014.
3. Tsang SM, Brown L, Lin K, Liu L, Piper K, O'Toole EA, Grose R, Hart IR, Garrod DR, Fortune F, *et al*: Non-junctional human desmoglein 3 acts as an upstream regulator of Src in E-cadherin adhesion, a pathway possibly involved in the pathogenesis of pemphigus vulgaris. J Pathol 227: 81-93, 2012.
4. Liu Q, Si T, Xu X, Liang F, Wang L and Pan S: Electromagnetic radiation at 900 MHz induces sperm apoptosis through bcl-2, bax and caspase-3 signaling pathways in rats. Reprod Health 12: 65, 2015.
5. Crowther CA, Aghajafari F, Askie LM, Asztalos EV, Brocklehurst P, Bubner TK, Doyle LW, Dutta S, Garite TJ, Guinn DA, *et al*; PRECISE Study Group: Repeat prenatal corticosteroid prior to preterm birth: a systematic review and individual participant data meta-analysis for the PRECISE study group (prenatal repeat corticosteroid international IPD study group: Assessing the effects using the best level of evidence) - study protocol. Syst Rev 1: 12, 2012.
6. Sun H, Zhou Y, Xiong H, Kang W, Xu B, Liu D, Zhang X, Li H, Zhou C, Zhang Y, *et al*: Prognosis of very preterm infants with severe respiratory distress syndrome receiving mechanical ventilation. Lung 193: 249-254, 2015.
7. Srivastava SK, Bhardwaj A, Arora S, Tyagi N, Singh S, Andrews J, McClellan S, Wang B and Singh AP: MicroRNA-345 induces apoptosis in pancreatic cancer cells through potentiation of caspase-dependent and -independent pathways. Br J Cancer 113: 660-668, 2015.
8. Takei Y, Okamoto S, Kawamura K, Jiang Y, Morinaga T, Shingyoji M, Sekine I, Kubo S, Tada Y, Tatsumi K, *et al*: Expression of p53 synergistically augments caspases-mediated apoptosis induced by replication-competent adenoviruses in pancreatic carcinoma cells. Cancer Gene Ther 22: 445-453, 2015.
9. Pradeep AR: Expression of key executioner of apoptosis caspase-3 in periodontal health and disease. J Investig Clin Dent 11: 356-358, 2011.
10. Swamy M, Suhaili D, Sirajudeen KN, Mustapha Z and Govindasamy C: Propolis ameliorates tumor nerosis factor-α, nitric oxide levels, caspase-3 and nitric oxide synthase activities in kainic acid mediated excitotoxicity in rat brain. Afr J Tradit Complement Altern Med 11: 48-53, 2014.
11. Tian F, Fu X, Gao J, Zhang C, Ning Q and Luo X: Caspase-3 mediates apoptosis of striatal cells in GA I rat model. J Huazhong Univ Sci Technolog Med Sci 32: 107-112, 2012.
12. Huang CS, Kawamura T, Peng X, Tochigi N, Shigemura N, Billiar TR, Nakao A and Toyoda Y: Hydrogen inhalation reduced epithelial apoptosis in ventilator-induced lung injury via a mechanism involving nuclear factor-kappa B activation. Biochem Biophys Res Commun 408: 253-258, 2011.
13. Merido D: Targeting BCL-2 to enhance vulnerability to therapy in estrogen receptor-positive breast cancer. Oncogene 8: 321-323, 2015.
14. Abdel Shakor AB, Atia M, Alshehri AS, Sobota A and Kwiatkowska K: Ceramide generation during curcumin-induced apoptosis is controlled by crosstalk among Bcl-2, Bcl-xL, caspases and glutathione. Cell Signal 27: 2220-2230, 2015.
15. Adefolaju GA, Theron KE and Hosie MJ: BAX/BCL-2 mRNA and protein expression in human breast MCF-7 cells exposed to drug vehicles-methanol and dimethyl sulfoxide (DMSO) for 24 hours. Niger Med J 56: 169-174, 2015.
16. Aliparasti MR, Alipour MR, Almasi S and Feizi H: Ghrelin administration increases the Bax/Bcl-2 gene expression ratio in the heart of chronic hypoxic rats. Adv Pharm Bull 5: 195-199, 2015.
17. Dagistan Y, Cukur S, Dagistan E and Gezici AR: Importance of IL-6, MMP-1, IGF-1 and BAX levels in lumbar herniated discs and posterior longitudinal ligament in patients with sciatic pain. World Neurosurg 84: 1739-1746, 2015.
18. Wright C: S-Nitrosylation of Bcl-2 negatively affects autophagy in lung epithelial cells. J Cell Biochem 8: 102-104, 2015.
19. Harazono Y, Kho DH, Balan V, Nakajima K, Zhang T, Hogan V and Raz A: Galectin-3 leads to attenuation of apoptosis through Bax heterodimerization in human thyroid carcinoma cells. Oncotarget 5: 9992-10001, 2014.
20. Aboutaleb N, Shamsaei N, Khaksari M, Erfani S, Rajabi H and Nikbakht F: Pre-ischemic exercise reduces apoptosis in hippocampal CA3 cells after cerebral ischemia by modulation of the Bax/Bcl-2 proteins ratio and prevention of caspase-3 activation. J Physiol Sci 65: 435-443, 2015.