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Effects and mechanisms of ambroxol inhalation (Mucosolvan®) in the treatment of neonatal pneumonia

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Neonatal pneumonia is the leading cause of mortality in children aged <5 years. Ambroxol (Mucosolvan®) is a mucolytic and secretolytic drug and belongs to the group of expectorants with anti-oxidative and anti-inflammatory effects. The purpose of the present study was to observe the effects and mechanisms of Mucosolvan® inhalation on neonatal pneumonia. Between January 2014 and October 2015, a total of 80 newborns with pneumonia were randomly divided into control and observation groups. While the patients in the control group were treated with conventional treatment only, those patients in the observation group were treated with Mucosolvan® in addition to the conventional treatment. The lung function index and serum inflammatory mediators were measured before and after treatment on days 1, 3 and 7. In the observation group, there was a significant increase in the lung function index as compared to the control group. Also, there was a significant decrease observed in the expression of inflammatory factors which in turn activated NF-κB pathway and cell apoptosis. The above findings had shown that Mucosolvan® improved lung function and exhibited good inflammatory response. In addition, we found that Mucosolvan® inhibited cell apoptosis and NF-κB pathway activation and effectively improved pulmonary functions.

1. Introduction

Neonatal pneumonia is a serious respiratory infection, and is considered as the most common infectious disease in neonates and is a significant cause of mortality. According to the World Health Organization (WHO), approximately 156 million new episodes of neonatal pneumonia that occur worldwide each year, of which developing countries account for 95% (Foote 2012; WHO/UNICEF). Among these cases, nearly 8.7% are life-threatening and require hospitalization (Islam et al. 2015; Jena 2014). Globally, neonatal pneumonia accounts for about 15% deaths and in 2013, there were about 935,000 children deaths less than 5 years old (Sathian et al. 2015). The major causes of this disease are: undernutrition, unsafe water and sanitation, air pollution and inadequate access to healthcare (Islam et al. 2015). Several studies indicated that bacterial pneumonia is primarily caused by *Streptococcus pneumoniae* and *Haemophilus influenzae* followed by *Staphylococcus aureus* and *Klebsiella pneumoniae* (Aydemir et al. 2016; Shen et al. 2010). In recent times, there are several approaches to determine the cause of pneumonia in developing countries including prospective hospital-based studies which use blood culture and percutaneous lung aspiration (Berti et al. 2013; Naderi et al. 2015).

The general treatment includes antibiotics for the management of neonatal sepsis, but there is an increase in the rate of resistance to antibiotics which may lead to fatalities (Zaidi et al. 2011). Thus, there is a need for a widely applicable treatment and preventive measures for the management of neonatal pneumonia.

Inflammatory response plays a key role in the induction of neonatal infections. Several studies suggested that neonatal brain damage and chronic lung disease can be predicted by the elevated levels of pro-inflammatory cytokines in cord blood and lung lavage (An et al. 2004; Wayock et al. 2013). The inflammatory response is initiated by the interaction between endothelial cells and leukocytes. The adhesion of leukocytes to endothelial cells is mediated by molecules such as intercellular cell adhesion molecule-1 (ICAM-1). However, in the presence of bacterial infections, there is an elevation in the levels of soluble ICAM-1 in neonates (Mahmoud et al. 2009). The expression of cytokines like interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α) and interleukin-1-beta (IL-1β) are also key mediators in the pathogenesis of infection (Balany and Bhandari 2015; Cao et al. 2012; Nwobodo et al. 2011).

At present, the treatment of neonatal pneumonia is focused on reduction of edema, inflammatory exudation and improvement of pulmonary ventilation function. Therefore, anti-inflammatory and expectorant treatment is essential for an effective treatment. This study mainly aimed at exploring the application of ambroxol inhalation (Mucosolvan®) in the treatment and potential mechanisms of neonatal pneumonia.

2. Investigations and results

2.1. Effects of Mucosolvan® on lung function index

A significant increase of PEF, FRC and TEF25% in the treatment group indicated that Mucosolvan® improves lung function index on days 1, 3, and 7 ($P<0.05$) when compared to control. There were no significant differences observed in the lung function index at baseline (Fig. 1A-1C).

2.2. Effects of Mucosolvan® on the expression of inflammatory factors

The expression of inflammatory factors, TNF-α, IL-6 and IL-10, was significantly ($P<0.05$) decreased from baseline to days 1, 3 and 7 following administration of Mucosolvan compared to the control group (Fig. 2A-2C).

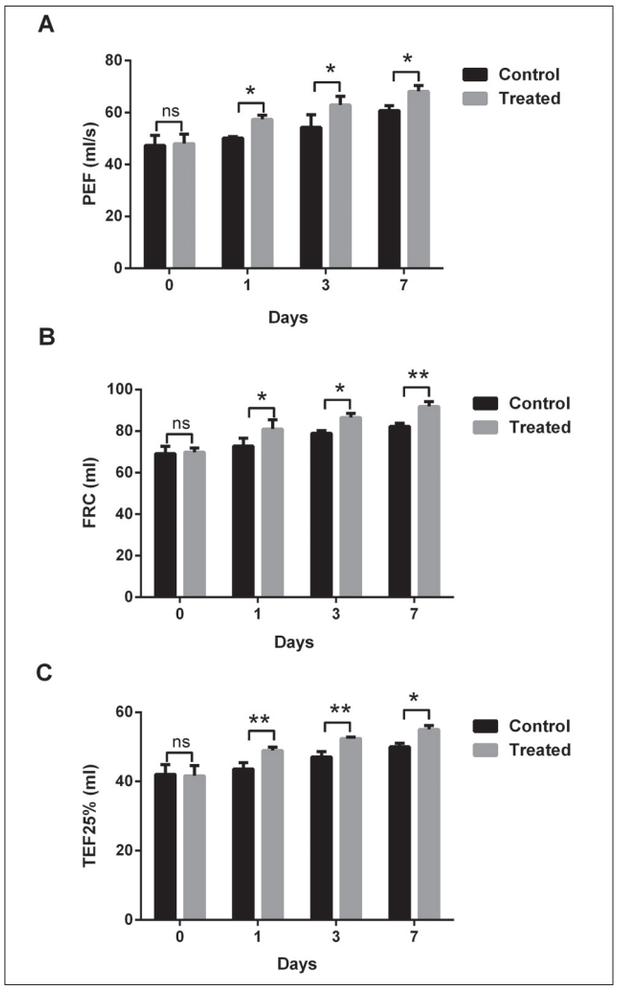

Fig. 1: Increase in PEF, FRC and TEF25% following Mucosolvan® treatment. A: PEF was significantly increased in the treated group from day 1, to day 3 till day 7 compared to that at day 0. B: FRC was significantly increased in the treated group from day 1, to day 3 till day 7 compared to that at day 0. C: TEF 25% was significantly increased in the treated group from day 1, to day 3 till day 7 compared to that at day 0. ns, not significant, * P<0.05 and ** P<0.01 compared to the control group. PEF: peak expiratory flow, FRC: Functional Residual Capacity, TEF25%: trachea-esophageal fistula 25%.

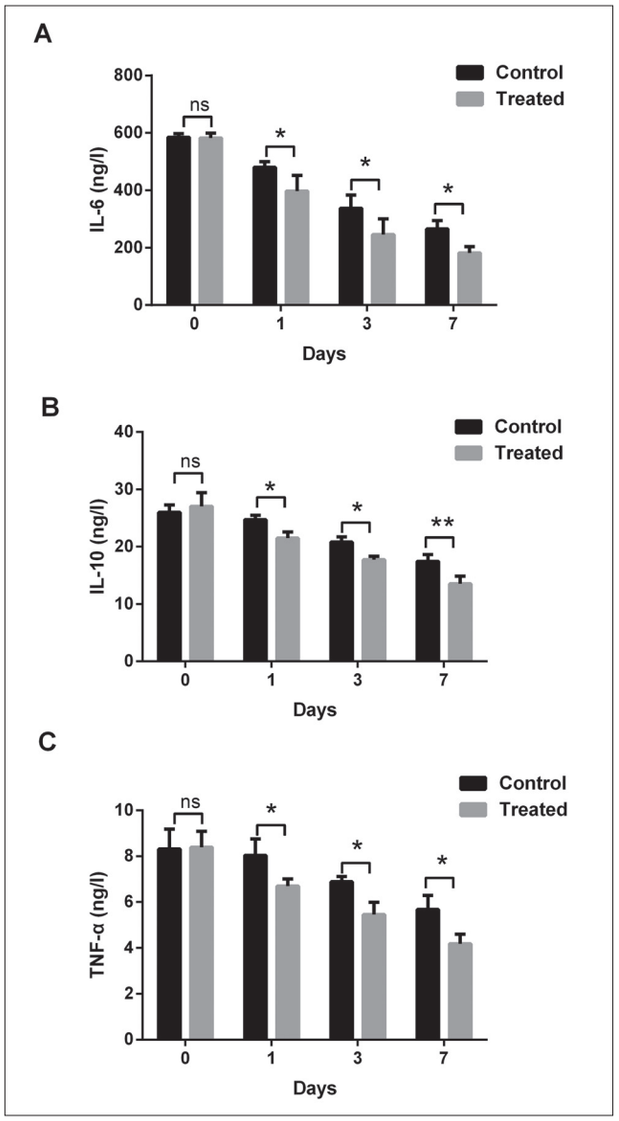

Fig. 2: Decrease in TNF-α, IL-6 and IL-10 following Mucosolvan® treatment. ns, not significant, * P<0.05 and ** P<0.01 compared to the control group. TNF-α: tumor necrosis factor alpha, IL-6: Interleukin-6, IL-10: Interleukin-10.

2.3. Effects of Mucosolvan® on cell apoptosis

Following Mucosolvan® treatment, the cell apoptosis rate in tissues was significantly decreased. It was also found that Mucosolvan® treatment significantly (P<0.05) decreased the expression of Bax but significantly (P<0.01) increased the expression of Bcl-2 moderately on days 1, 3, and 7 (Fig. 3A and 3B). Moreover, a significant decrease (P<0.05) in the percentage of TUNEL positive neurons by day 7 was also seen. These results suggested that Mucosolvan® decreased the apoptosis rate.

2.4. Effects of Mucosolvan® on NF-κB pathway

Western blotting results showed that the expression levels of the inhibitors IκBa and p-NF-κB were significantly decreased on day 7 (P<0.01) after the Mucosolvan® treatment, indicating that it activated the NF-κB pathway (Fig. 4A and 4B).

3. Discussion

In the present study, we explored the effects and mechanisms of ambroxol (Mucosolvan®)
inhalation in neonatal pneumonia. Despite advances in the healthcare for pediatrics, neonates are still at a higher risk of developing infections when compared to older children and adults who can resist bacterial infections. Several studies indicated that this process was mediated by distinct inflammatory cytokines (Rautiainen et al. 2014).

Ambroxol (Mucosolvan®), a metabolite of bromhexine, has several pharmacological properties such as surfactant stimulation, anti-inflammation, anti-oxidant and local anesthetic effects in addition to the mucokinetic and mucociliary effects of the parent compound (Chen 2014). Its anti-inflammatory property and surfactant stimulatory properties have paved its way in the management of obstructive airway disorders (Gupta 2010). It has been shown to elucidate protective effects in acute lung injury models, decreased levels of lipopolysaccharide induced lung hemorrhage, edema, exudation, infiltration with neutrophils and release of cytokines in animal studies (Su et al. 2004). In a study by Zhi et al. (2011) showed that Mucosolvan® blocked paraquat-induced rise in oxidant stress markers, cytokines (like TNF-α, TGF-β1, etc.) and tissue inhibitor of metalloproteinase-1, total inflammatory cell count, hydroxyproline content and collagen I and III messenger ribonucleic acid (mRNA) while preserving the superoxide dismutase (SOD) and glutathione system (GSH-PX) activities in rat lungs (Zhi et al. 2011). Several *in vitro* studies indicated that Mucosolvan® blocked the release of several oxidant stress markers, cytokines,

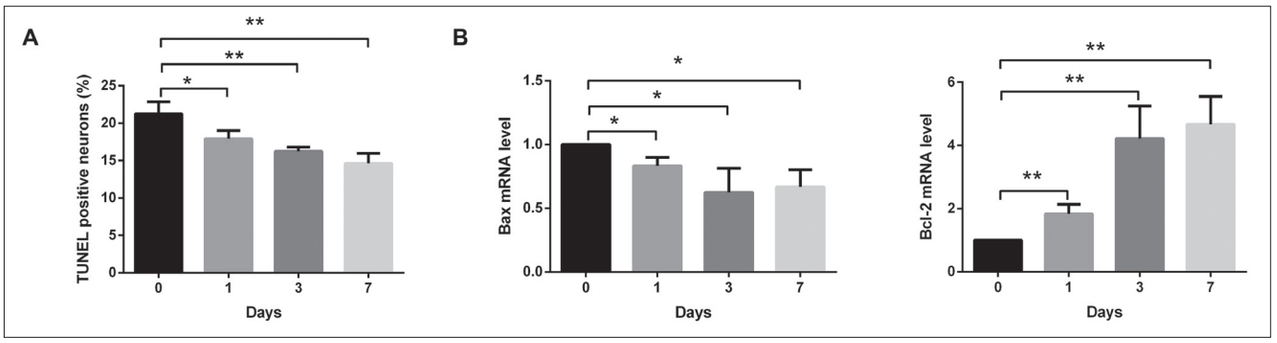

Fig. 3: Effect of Mucosolvan® cell apoptosis. * P<0.05 and ** P<0.01 compared to the control group.

MPO, nitric oxide and collagen I and III mRNA while it preserves SOD and GSH-PX activities (Gupta 2014). In our study, we used Mucosolvan® as an anti-oxidant to inhibit lung damage caused by oxygen radicals and used PEF, FRC and TEF25% levels as lung function index. Our findings supported the fact that Mucosolvan® treatment is able to improve lung function index. Our results have shown a significant improvement in lung function from baseline to days 1, 3 and 7.

The key role of TNF-α, IL-1β, IL-6, and IL-8 in the pathogenesis of neonatal pneumonia was well established by previous studies (Ishida et al. 2006; Zhi et al. 2011). It is also known that neonates have reduced capacity to produce IL-6 (Abdollahi et al. 2012; Yu et al. 2013), but in the presence of an infection, there are high levels of IL-6 in plasma (Dai and Chan 2014; Zheng et al. 2011). In the present study, we determined anti-inflammatory properties by measuring the expression of factors like TNF-α, IL-6 and IL-10 using ELISA and RT–qPCR. We found a significant decrease in the levels of these inflammatory factors following treatment with Mucosolvan®. It has been shown that an abundance of oxygen free radicals lead to severe inflammatory response (Closa and Folchpuy 2004). The use of antioxidants or scavengers of oxygen free radicals like Mucosolvan® may reduce the levels of some inflammatory mediators, cytokines, growth factors or cell death (Mukhopadhyay et al. 2012). We used TUNEL assay to determine apoptosis and our results are inline with previous studies (Lebon et al. 2015) which indicated that Mucosolvan® decreased the cell death rate that may be attained by inhibition of activation process, protein kinases and calcium transport.

The strengths of our study are the population base and ascertainment of the role of Mucosolvan® in neonatal pneumonia. The data obtained from this study indicated that Mucosolvan® protects lung function, decreases inflammatory response and cell apoptosis. However, there are certain inadequacies in our study and one being a lack of standard measures of response to a bronchodilator in an infant pulmonary function test. We have used >25% as an improvement in the lung function following treatment for positive response. This required standard criteria to support our findings.

In conclusion, we demonstrated a protective lung function and decreased inflammatory response by Mucosolvan® in neonatal pneumonia. In addition, we have also found that Mucosolvan® inhibited cell apoptosis and NF-κB pathway activation. However, more studies with large numbers of patients are needed to confirm Mucosolvan's abilities to improve pulmonary function.

4. Experimental

4.1. Patients and treatment

A total of 80 newborns with pneumonia were randomly divided into control and observation group between January 2014 and October 2015. Patients in the control and observation group were treated with conventional treatment and Mucosolvan® (7.5 mg dissolved in 5% GS, twice a day for 7 days) plus conventional treatment, respectively. Conventional treatments included anti-infection, wet airway, regulation of acid-base imbalance and sputum suction. All the patients parents were informed with consent according to the guidelines in the commitment of The Second Affiliated Hospital and Yuying Children's Hospital of Wenzhou Medical University.

4.2. ELISA and lung function index

The expression of IL-2, IL-6, and IL-10 were analyzed using ELISA pre- and post-treatment on days 1, 3 and 7. In addition, lung function index including peak expiratory flow (PEF), functional residual capacity (FRC) and trachea-esophageal fistula (TEF25%) were analyzed.

4.3. Real-time quantitative PCR (RT–qPCR)

RT–qPCR was performed using primers to detect Bax, Bcl-2 and housekeeping gene β-actin (ACTB) on a ABI PRISM 7700 Sequence Detection System using pre-developed TaqMan assay reagents (Applied Biosystems, Foster City, CA). Quantitative analysis of gene expression was performed using the comparative threshold cycle (CT) method (CT, threshold cycle number, is the minimum number of cycles needed before the product can be detected). The arithmetic formula for the CT method is given by the difference in threshold cycles for a target and an endogenous reference (e.g., housekeeping gene β-actin). The amount of target normalized to an endogenous reference and relative to a calibration normalized to an endogenous reference is given by $2^{-\Delta\Delta CT}$.

4.4. Apoptosis assay

Apoptosis in lung tissue sections was measured using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) detection kit (Roche Applied Science, Indianapolis, IN, USA). The slides were then analyzed by fluorescence microscopy and images were captured using a Photometrix digital camera mounted on a Nikon laser microscope with MetaMorph imaging software (MDS Analytical Technologies, Downingtown, PA).

4.5. Western blot

The procedure was performed according to the standard protocols for western blotting analysis. The prepared cells were first trypsinized and harvested, then washed once with PBS and re-suspended in cell lysis buffer (PBS with 1% Triton X-100 and protease inhibitors) for western blotting. After brief sonication, cell lysates were centrifuged at 13,000 rpm for 5 min. Protein concentration was determined and its equivalent amounts of lysate was added to an equal volume of 2 × Laemmli buffer and boiled for 10 min. Finally, proteins were detected by SuperSignal enhanced chemiluminescence development (ECL) (Thermo Scientific Pierce) reagent and exposed to films (Kodak). The protein level quantification was carried out by Image J.

4.6. TUNEL assay

The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay was performed using the *in-situ* cell death detection kit (Roche Diagnostics Corporation, Indianapolis, IN, USA). After transfection, the cells were seeded at 5×10^3 cells per well in 14-well plates at 24 h. The cells were then treated with 0.05 ppc paclitaxel for 1 h and fixed using 4% paraformaldehyde and incubated in 0.1% TritonX-100 for 2 min on ice and washed. After washing, the TUNEL reaction mixture was added to each well and incubated for 1 h at 37 °C in the dark. The cells were stained using DAPI (4, 6-diamidino-2-phenylindole, Dojindo Molecular Technologies, Inc., Japan). Fluorescence images were obtained at an excitation wavelength of 488 nm for FITC-labeled nanoparticles using NISE elements F 2.20 imaging software (Nikon, Tokyo, Japan).

4.7. Statistical analysis

All the data are presented as mean±SEM. Statistical significance was calculated using the two-tailed Fisher's exact t-test or one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multigroup comparisons as appropriate. A *P*-value of <0.05 was considered as statistically significant.

Conflicts of interest: None declared.

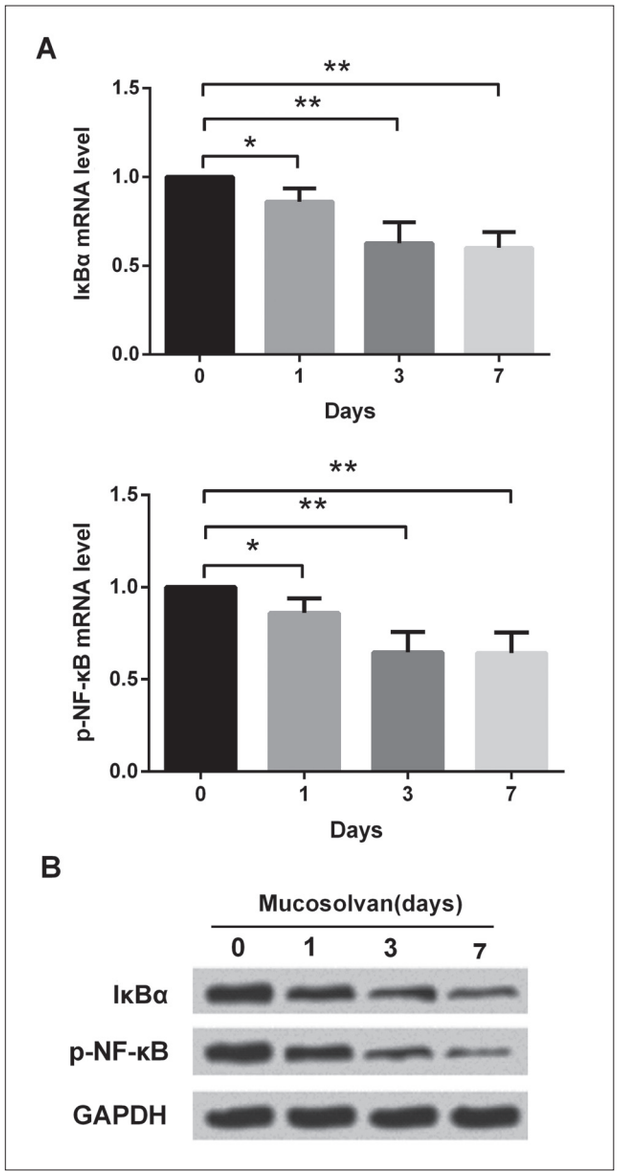

Fig. 4: Effect of Mucosolvan® on NF-κB pathway. * P<0.05 and ** P<0.01 compared to the control group.

References

Abdollahi A, Shoar S, Nayyeri F, Shariat M (2012) Diagnostic value of simultaneous measurement of procalcitonin, interleukin-6 and hs-CRP in prediction of early-onset Neonatal sepsis. Mediterranean J Hematol Infect Dis 4: e2012028.

An H, Nishimaki S, Ohyama M, Haruki A, Naruto T, Kobayashi N, Sugai T, Kobayashi Y, Mori M, Seki K (2004) Interleukin-6, interleukin-8, and soluble tumor necrosis factor receptor-I in the cord blood as predictors of chronic lung disease in premature infants. Am J Obst Gynecol 191: 1649-1654.

Aydemir Y, Aydemir Ö, Pekcan S, Özdemir M (2016) Value of multiplex PCR to determine the bacterial and viral aetiology of pneumonia in school-age children. Paediatr Int Child Health 1-6.

Balany J, Bhandari V (2015) Understanding the impact of infection, inflammation, and their persistence in the pathogenesis of bronchopulmonary dysplasia. Frontiers Med 2: 90.

Berti E, Galli L, De Martino M, Chiappini E (2013) International guidelines on tackling community – acquired pneumonia show major discrepancies between developed and developing countries. Acta Paed Suppl. 102: 4-16.

Cao YZ, Tu YY, Chen X, Wang BL, Zhong YX, Liu MH (2012) Protective effect of Ulinastatin against murine models of sepsis: inhibition of TNF-α and IL-6 and augmentation of IL-10 and IL-13. Exp Toxicol Pathol 64: 543-547.

Chen H (2014) [Study on the efficacy of Mucosolvan combined with chymotrypsin aerosol inhalation in the treatment for bronchial pneumonia among children]. Medical Recapitulate 2014: 18.

Closa D, Folchpuy E (2004) Oxygen free radicals and the systemic inflammatory response. IUBMB Life 56: 185-191.

Dai H, Cao B (2014) [Relationship of procalcitonin, interleukin-6 and C-reactive protein with neonatal bacterial infection types and severity]. Chin J Exp Clin Infect Dis 2014: 06.

Foote E (2012) Health impact of locally-produced, improved ceramic cookstoves on children in rural Western Kenya. Emory University.

Gupta PR (2010) Ambroxol - Resurgence of an old molecule as an anti-inflammatory agent in chronic obstructive airway diseases. Lung India 27: 46-48.

Gupta PR (2014) Ambroxol hydrochloride in the management of idiopathic pulmonary fibrosis: Clinical trials are the need of the hour. Lung India 31: 43-46.

Ishida Y, Takayasu T, Kimura A, Hayashi T, Kakimoto N, Miyashita T, Kondo T (2006) Gene expression of cytokines and growth factors in the lungs after paraquat administration in mice. Leg Med (Tokyo) 8: 102-109.

Islam MMZ, Hossain MM, Mamun MAA, Mahmud S, Yasmeen BN (2015) Risk factors which affect the change of antibiotics in neonatal pneumonia observed in a tertiary care hospital. Northern Int Med Coll 6: 21.

Jena M (2014) Effectiveness of information booklet on knowledge & practice about prevention of pneumonia among mothers of under five children. J Nursing Health Sci 3: 25-30.

Lebon C, Villalpando RG, El ZI, Jaadane I, Beharcohen F, Torriglia A (2015) On the use of an appropriate TUNEL assay to identify apoptotic cells. Anal Biochem 480: 34-41.

Mahmoud S, Maklad S, Abo Elmagd EK (2009) Soluble intercellular adhesion molecule-1 (slCAM-1) for early diagnosis of neonatal infections. Egypt J Immunol 16; 83.

Mukhopadhyay P, Horváth B, Zsengellèr Z, Bátkai S, Cao Z, Kechrid M, Holovac E, Erdélyi K, Tanchian G, Liaudet L (2012) Mitochondrial reactive oxygen species generation triggers inflammatory response and tissue injury associated with hepatic ischemia-reperfusion: therapeutic potential of mitochondrially targeted antioxidants. Free Rad Biol Med 53: 1123-1138.

Naderi H, Sheybani F, Sarvghad M, Meshkat Z, Jabbari NM (2015) Etiological diagnosis of community-acquired pneumonia in adult patients: a prospective hospital-based study in Mashhad, Iran. Jundishapur J Microbiol 8: e22780.

Nwobodo NN, Okonkwo PO, Igwe SA (2011) Role of tumour necrosis factor (TNF) and glycosylphosphatidylinositol (GPI) as important mediators in the pathogenesis of malaria infection. Biosci Biotechnol Res Asia 8: 113-117.

Rautiainen L, Ince S, Pavare J, Grope I, Tretjakovs P, Gardovska D (2014) Levels of inflammatory cytokines and chemokines in hospitalized children with sepsis and opneumonia. Acta Chirurg Latv 13: 43-46.

Sathian B, De A, Simkhada P, Malla K, Ghosh A, Basnet S, Roy B, Banerjee I, Supram HS, Devkota S (2015) Time trend of pneumonia in under five children of Nepal. Am J Publ Health Res 3: 27-30.

Shen H, Liu X, Tan X (2010) [Distribution and drug resistance of pathogenic bacteria in 206 children with bacterial pneumonia]. Practical Preventive Medicine 2010: 02.

Su X, Wang L, Song Y and Bai C (2004) Inhibition of inflammatory responses by ambroxol, a mucolytic agent, in a murine model of acute lung injury induced by lipopolysaccharide. Intensive Care Med 30: 133-140.

Wayock C, Nigrini E, Graham E, Johnston MV, Burd I (2013) Magnesium in inflammation-associated fetal brain injury. In: Watson RR, Preedy VR, Zibady S (eds.) Magnesium in Human Haelth and Disease, New York, pp. 231-240.

WHO/UNICEF (2013) Ending preventable child deaths from pneumonia and diarrhoea by 2025. The integrated Global Action Plan for Pneumonia and Diarrhoea (GAPPD). Geneva, Switzerland.

Yu SU, Huang T, Wei HB (2013) Clinical value of procalcitonin and interleukin-6 in early diagnosis of neonatal pneumonia. China J Modern Med 23: 100-103.

Zaidi AK, Ganatra HA, Syed S, Cousens S, Lee AC, Black R, Bhutta ZA, Lawn JE (2011) Effect of case management on neonatal mortality due to sepsis and pneumonia. BMC Public Health 11: 1-15.

Zheng KJ, Yang XF, Chen J, Huang HJ, Chen JG (2011) The level of inflammatory mediators into plasma in ventilator associated pneumonia of neonates. J Clin Med Pract 2011 (3): 51-53.

Zhi QM, Yang LT, Sun HC (2011) Protective effect of ambroxol against paraquat-induced pulmonary fibrosis in rats. Intern Med 50: 1879-1887.