ISSN 2186-3644 Online ISSN 2186-361X

IRDR

Intractable & Rare Diseases Research

Volume 4, Number 2
May, 2015

IRCA BSSA
IACMHR

www.irdrjournal.com

IRDR
Intractable & Rare Diseases Research

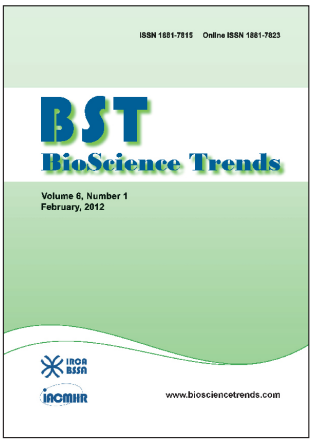
ISSN: 2186-3644
Online ISSN: 2186-361X
CODEN: IRDRA3
Issues/Year: 4
Language: English
Publisher: IACMHR Co., Ltd.

Intractable & Rare Diseases Research is one of a series of peer-reviewed journals of the International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) Group and is published quarterly by the International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) and supported by the IRCA-BSSA, Shandong Academy of Medical Sciences, and Shandong Rare Disease Association.

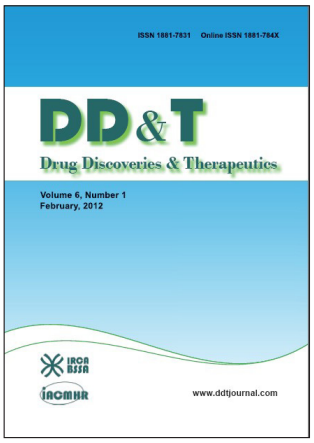
Intractable & Rare Diseases Research devotes to publishing the latest and most significant research in intractable and rare diseases. Articles cover all aspects of intractable and rare diseases research such as molecular biology, genetics, clinical diagnosis, prevention and treatment, epidemiology, health economics, health management, medical care system, and social science in order to encourage cooperation and exchange among scientists and clinical researchers.

Intractable & Rare Diseases Research publishes Original Articles, Brief Reports, Reviews, Policy Forum articles, Case Reports, News, and Letters on all aspects of the field of intractable and rare diseases research. All contributions should seek to promote international collaboration.

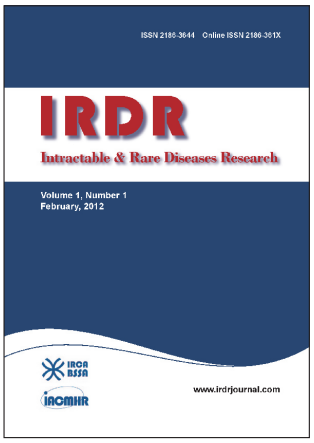
IRCA-BSSA Group Journals



ISSN: 1881-7815
Online ISSN: 1881-7823
CODEN: BTIRCZ
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.
www.biosciencetrends.com



ISSN: 1881-7831
Online ISSN: 1881-784X
CODEN: DDTRBX
Issues/Year: 6
Language: English
Publisher: IACMHR Co., Ltd.
www.ddtjournal.com



ISSN: 2186-3644
Online ISSN: 2186-361X
CODEN: IRDRA3
Issues/Year: 4
Language: English
Publisher: IACMHR Co., Ltd.
www.irdrjournal.com

Intractable & Rare Diseases Research

Editorial and Head Office

Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku,
Tokyo 112-0003, Japan

Tel: +81-3-5840-9968, Fax: +81-3-5840-9969
E-mail: office@irdrjournal.com
URL: www.irdrjournal.com

Editorial Board

CONTENTS

Volume 4, Number 2, 2015

Reviews

Original Articles

Case Reports

CONTENTS (*Continued*)

(*This journal was partially supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science.*)

Mini-Review

DOI: 10.5582/irdr.2015.01009

Diagnosis of idiopathic pulmonary fibrosis: Current issues

Rajendra Prasad[1,*], Nikhil Gupta[2], Abhijeet Singh[1], Pawan Gupta[1]

[1] *Department of Respiratory Medicine, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India;*
[2] *Department of Internal Medicine, Era Medical College, Lucknow, India.*

Summary **Idiopathic pulmonary fibrosis (IPF) accounts for the majority of lung diseases classified as idiopathic interstitial pneumonia (IIP). It is considered to be lethal because prognosis is very poor and far worse than other types of IIP. An early and accurate diagnosis of IPF is critical. The diagnostic process is complex and requires a multidisciplinary approach involving a pulmonologist, radiologist and pathologist.**

Keywords: Idiopathic pulmonary fibrosis, idiopathic interstitial pneumonia, diagnosis

1. Introduction

Idiopathic interstitial pneumonia (IIP) makes up a heterogeneous group of diseases. The most common and most lethal type of IIP is idiopathic pulmonary fibrosis (IPF) that accounts for 55% of lung diseases classified as IIP (*1-3*). Louis Hamman and Arnold Rich published a paper describing diffuse interstitial fibrosis of the lung in 1944 (*4*). They described thickening of the alveolar interstitium and an area of dense fibrotic scar tissue within the lung. Liebow and Carrington heralded the modern era of interstitial lung disease histo-pathology with the notion that IIP could be split into separate pathological subtypes in 1969 (*5*). In 2002, a panel of experts sponsored jointly by the American Thoracic Society (ATS) and the European Respiratory Society (ERS) released an official statement for the purpose of providing a new and comprehensive classification of IIP that considered all clinical, radiographic and pathological features (*1*). In the recent revision of the IIP classification, IPF is classified under major IIP and has been sub-categorized under chronic fibrosing interstitial pneumonias. The alternative terminology "cryptogenic fibrosing alveolitis" has been eliminated, leaving IPF as the sole clinical term for this diagnosis (*6*). The prognosis for individuals with IPF is poor, far worse than nonspecific interstitial pneumonia and other IIP, with a 5 years survival rate that is worse than several types of cancer (*7,8*). An early and accurate diagnosis of IPF is critical. Diagnosis of IPF requires precision and a multidisciplinary approach involving pulmonologists, radiologists and pathologists (*2*). This article aims to review the current issues in the diagnosis of IPF.

**Address correspondence to:*
Dr. Rajendra Prasad, Department of Respiratory Medicine, Vallabhbhai Patel Chest Institute, University of Delhi, Delhi, India.
E-mail: rprasadkgmc@gmail.com; rprasaddirvpci@gmail.com

2. Definition

IPF is defined as a specific form of chronic, progressive fibrosing interstitial pneumonia characterized by idiopathic origin, occurrence primarily in older adults, exclusively pulmonary involvement and pattern of Usual Interstitial Pneumonia (UIP) proven by histopathology and/or radiology. The confirmation of diagnosis of IPF requires the exclusion of other idiopathic interstitial pneumonias (IIP's) and interstitial lung disease associated with environment exposure, drugs, or systemic disease (*9-11*).

3. Diagnosis

3.1. *Clinical Features*

The diagnosis of IPF should be suspected in those adult patients with older age typically in the sixth and seventh decades. Its occurrence is rare in less than fifty years of age provided there could be manifestation of overt features of an underlying connective tissue disease that was sub-clinical at the time of IPF diagnosis. (*18,19*). The clinical features commonly associated with IPF are unexplained chronic exertional dyspnea, dry cough, bibasilar end-inspiratory crackles also known as Velcro crackles, and finger clubbing (found in 60% of cases).

(*12-17*). IPF is more common in men than women and the majority of patients have a history of cigarette smoking (*10-15*).

3.2. *Radiological Features*

The revised guidelines identify the high-resolution computed tomography (HRCT) features of IPF as UIP, possible UIP and inconsistent with UIP patterns (*2*). The features of a classical UIP pattern on HRCT require *i*) the presence of sub-pleural abnormalities with lower lobe predominance *ii*) reticular abnormality, *iii*) honeycombing with or without traction bronchiectasis and *iv*) exclusion of other features inconsistent with UIP pattern (upper or middle lobe predominance, peribronchovascular involvement, extensive ground glass abnormality, profuse micronodules, discrete multiple cysts other than honeycombing, extensive mosaic pattern and/or air trapping, or sub-pleural or basal consolidation (*20-22*). The presence of honeycombing with or without traction bronchiectasis can make then the diagnosis difficult using HRCT alone. Further workup is required for diagnosis if HRCT scan is showing features of possible and/or inconsistent UIP patterns (*2*). The revised IPF guidelines emphasize the analysis of HRCT. However, the major problem in clinical practice is the classification of patients with a predominantly basal and sub-pleural distribution of reticular abnormalities typical of IPF, but without honeycombing on HRCT. The current guidelines have been unfortunate in categorizing those patients with IPF having traction bronchiectasis, co-existing emphysema and fibrosis (*23*), and/or those above 60 years of age (*24*). Further expertise is needed in distinguishing typical and atypical HRCT appearances of IPF, as there is significant inter-observer variation among clinicians (*25*). Expert interpretation of HRCT data and the identification of honeycombing are important landmarks for radiological diagnosis of IPF (*26*).

3.3. *Histopathological features*

The diagnosis of definite IPF is uncertain in at least one-third of cases by HRCT thorax alone (*27,28*). It is recommended currently that a surgical lung biopsy should be performed when the diagnosis of definite IPF is uncertain even with HRCT thorax (*2*). The histopathological pattern of UIP is characteristically inhomogeneous, bilateral, involving lower lobes, predominantly sub-pleural occurrence and patchy in appearance (*2*). For the definitive diagnosis of UIP, features are *i*) marked fibrosis with or without honeycombing in a predominantly sub-pleural/ paraseptal distribution, *ii*) patchy parenchymal fibrosis and *iii*) presence of fibroblast foci. Probable UIP pattern includes *i*) evidence of marked fibrosis with or without honeycombing, *ii*) absence of either patchy involvement or fibroblastic foci, but not both *iii*) absence of features against a diagnosis of UIP suggesting an alternative diagnosis *iv*) honeycombing changes only. Criteria for possible UIP pattern are *i*) patchy or diffuse parenchymal fibrosis with or without interstitial inflammation, *ii*) absence of other criteria for UIP, *iii*) absence of features against a diagnosis of UIP. Features not supporting UIP pattern are *i*) hyaline membrane, *ii*) organizing pneumonia, *iii*) granulomas, *iv*) marked interstitial inflammatory cells, *v*) predominantly airway centered changes, *vi*) other features suggestive of an alternative diagnosis.

3.4. *Other investigations*

3.4.1. *Bronchoalveolar lavage (BAL)*

The most important application of BAL is to increase the index of suspicion for alternative disorders in patients with suspected IPF. When evaluating such individuals, BAL is useful in excluding other conditions, especially Chronic Hypersensitivity Pneumonitis (HP), for which a diagnosis is suggested by lymphocytosis > 40% (*2*). Current evidence recommends that BAL cellular analysis should not be performed routinely in the diagnostic evaluation of a majority of patients with IPF as there is unclear evidence regarding diagnostic specificity (2).

3.4.2. *Transbronchial lung biopsy (TBB)*

Transbronchial lung biopsy (TBB) has been shown to be useful in establishment of diagnosis of diseases with predominantly bronchocentric involvement such as sarcoidosis that can mimic IPF by having a UIP pattern on HRCT in rare instances (*26,29-30*). The sensitivity and specificity are unknown for TBB in diagnosis of IPF even when the UIP pattern is demonstrated in biopsy material (*2,31*). Therefore, TBB is recommended in a minority of individuals (*2*).

3.4.3. *Serology*

Serological testing for connective tissues diseases is now considered to be part of routine diagnostic workup of IPF in most patients whether manifestation of connective tissue disorders exist or not. Serological evaluation includes predominantly rheumatoid factor (RF), anti-cyclic citrullinated peptide (anti-CCP) and anti-nuclear antibody titer (ANA) but other serological tests such as anti-synthetase antibodies (*e.g*. Jo-1), creatine kinase and aldolase, Sjogren's antibodies (SS-A, SS-B), and scleroderma antibodies (Scl-70, PM-1) can be indicated in appropriate clinical cases. Careful screening is required for exclusion of other connective tissues disorders with these clinical features such as arthritis, Raynaud's phenomenon, skin changes, abnormal

esophageal motility before commitment of diagnosis of IPF as few patients can have mildly positive antinuclear antibody titer and/or rheumatoid factor levels without any other clinical features of connective tissue. In the absence of additional serologic or clinical evidence to support a connective tissue diagnosis, the diagnosis of IPF is appropriate. Serial serologic and clinical evaluation monitoring is essential for subsequently confirming the development of a connective tissue disease associated ILD thereby requiring revision of the diagnosis (*2*).

3.4.4. *Biomarkers*

Identification of biomarkers has been an area of new research interest for diagnosis of IPF. They may be useful to identify patients at high risk of progression of disease apart from diagnosis. These biomarkers are estimated in serum as well as BAL. High levels of epithelial or macrophage-related proteins such as SP-A, SP-D, KL-6 (Krebs von den Lungen-6), CCL18 (chemokine ligand-18), and MMP-7 (matrix metalloproteinase-7) are found in IPF patients distinguishing from other IIP's (*32-37*). Further validation is required for their associations in the near future.

4. Evolution of diagnosis of IPF over the years

The diagnosis of IPF was established on the basis of the presence of all four major criteria and three out of four minor criteria over the decade that was the era before surgical lung biopsy (SLB) gained much importance (*1,9*). The four major criteria were *i*) exclusion of other known cause of ILD, such as medication exposure, environmental or occupational exposure and connective tissue disorders; *ii*) abnormal spirometric findings such as reduced forced vital capacity, with an increased ratio of forced expiratory volume in 1 second/forced vital capacity (evidence of restrictive pattern) and impaired gas exchange parameters including increased alveolar-arterial oxygen tension difference with rest or exercise or decreased diffusing capacity of the lung for carbon monoxide; *iii*) Bilateral reticular abnormalities with basal predominance and minimal ground glassing on HRCT thorax scan; and *iv*) TBB or BAL showing inconclusive findings to support diagnosis. The four minor criteria were *i*) age of onset more than 50 years, *ii*) insidious onset of otherwise unexplained dyspnea, *iii*) duration of illness of more than 3 months, and *iv*) dry or "velcro" bibasilar, inspiratory crackle. However, this set of framed criteria is observed to have significant limitations particularly concerning the four minor criteria. The early diagnosis of IPF can be missed in that subset of patients less than 50 years. Further, some IPF patients may initially present with acute symptomatic exacerbations rather than having insidious onset. Moreover, occurrence of co-morbid factors such as pre-existing pulmonary fibrosis and/or smoking related lung damage can make diagnosis of IPF difficult in patients with disease duration of ≥ 3 months. Similarly, the presence of crackles may not be evident for establishment of diagnosis of IPF.

The diagnostic guideline published in 2000 for IPF was later updated in 2011 by an international collaboration of the ATS and ERS, as well as the Japanese Respiratory Society (JRS) and the Latin American Thoracic Association (ALAT) in view of consideration of these limitations and recently gained importance of the multi-disciplinary approach of radiology and pathology subsets. (*2*) The diagnostic criteria were revised and are summarized: *i*) Exclusion of other known causes of ILD (domestic and occupational environmental exposure, connective tissue disease and drug toxicities) as mentioned before; *ii*) the presence of a definite UIP pattern on the HRCT scans in individuals not requiring further confirmation of diagnosis by SLB; and *iii*) the lack of definite UIP pattern and presence of other patterns on the HRCT scan requiring further confirmation by SLB. It can be stated that the presence of a classical UIP pattern on the HRCT scans is sufficient for the diagnosis of IPF in an appropriate clinical setting without SLB.

5. Conclusion

An early and accurate diagnosis of IPF is critical. The clinician has to rely on clinical, laboratory, radiologic, and/or pathologic data for establishment of diagnosis of this disease. An adult with unexplained exertional dyspnea with cough, bibasilar inspiratory crackles and clubbing in the presence of classical UIP pattern on HRCT is sufficient for the diagnosis of IPF. Other investigation like BAL, TBB and serological study should not be performed in the diagnostic evaluation of IPF in the majority of individuals but may be appropriate for a minority. A multidisciplinary approach is the mainstay to improve the diagnostic yield of IPF early and accurately.

References

1. American Thoracic Society, European Respiratory Society. American Thoracic Society/European Respiratory Society InternationalMultidisciplinary Consensus Classification of the Idiopathic Interstitial Pneumonias. This joint statement of the American Thoracic Society (ATS), and the EuropeanRespiratory Society (ERS) was adopted by the ATS board of directors, June 2001 and by the ERS Executive Committee, June 2001. Am J Respir Crit Care Med. 2002; 165:277-304.
2. Raghu G, Collard HR, Egan JJ, *et al*. An official ATS/ERS/JRS/ALAT statement: Idiopathic pulmonary fibrosis: Evidence-based guidelines for diagnosis and management. Am J Respir Crit Care Med. 2011; 183:788-824.
3. Meltzer EB, Noble PW. Idiopathic pulmonary fibrosis.

Orphanet Rare Dis. 2008; 3:8.
4. Hamman L, Rich AL. Acute diffuse interstitial fibrosis of lung. Bull John Hopkins Hospital. 1944; 74:177-212.
5. Liebow AA, Carrington CB. The interstitial pneumonias. In: Simon M, Potchen EJ, LeMay M, editors. Frontiers of pulmonary radiology, 1st edition, Grune & Stratton, NY, USA, 1969; pp. 102-141.
6. Travis WD, Costabel U, Hansell DM, *et al*. An Official American Thoracic Society/European Respiratory Society Statement: Update of the international multidisciplinary classification of the idiopathic interstitial pneumonias. Am J Respir Crit Care Med. 2013; 188:733-748.
7. Bjoraker JA, Ryu JH, Edwin MK, Myers JL, Tazelaar HD, Schroeder DR, Offord KP. Prognostic significance of histopathologic subsets in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 1998; 157:199-203.
8. American Cancer Society. Cancer Facts and Figures 2009. Atlanta, American Cancer Society, GA, USA, 2010.
9. American Thoracic Society. Idiopathic pulmonary fibrosis: Diagnosis and treatment. International consensus statement. American Thoracic Society (ATS), and the European Respiratory Society (ERS). Am J Respir Crit Care Med. 2000; 161(2 Pt 1):646-664.
10. Iwai K, Mori T, Yamada N, Yamaguchi M, Hosoda Y. Idiopathic pulmonary fibrosis. Epidemiologic approaches to occupational exposure. Am J Respire Crit Care Med. 1994; 150:670-675.
11. Coultas DB, Zumwalt RE, Black WC, Sobonya RE. The epidemiology of interstitial lung disease. Am J Respir Crit Care Med. 1994; 150:967-972.
12. Douglas WW, Ryu JH, Schroeder DR. Idiopathic pulmonary fibrosis: Impact of oxygen and colchicines, prednisone, or no therapy on survival. Am J Respir Crit Care Med. 2000; 161:1172-1178.
13. King TE Jr, Tooze JA, Schwarz MI, Brown KR, Cherniack RM. Predicting survival in idiopathic pulmonary fibrosis: Scoring system and model. Am J Respir Crit Care Med. 2001; 164:1171-1181.
14. Gribbin J, Hubbard RB, Le Jeune I, Smith CJ, West J, Tata LJ. Incidence and mortality of idiopathic pulmonary fibrosis and sarcoidosis in the UK. Thorax. 2006; 61:980-985.
15. Scott J, Johnston I, Britton J. What cause cryptogenic fibrosing alveolitis? A case- control study of environmental exposure to dust. BMJ. 1990; 301:1015-1017.
16. Mannino DM, Etzel RA, Parrish RG. Pulmonary fibrosis deaths in the United States, 1979-1991. An analysis of multiple-cause mortality data. Am J Respir Crit Care. 1996; 153:1548-1552.
17. Raghu G, Freudenberger TD, Yang S, Curtis JR, Spada C, Hayes J, Sillery JK, Pope CE 2nd, Pellegrini CA. High prevalence of abnormal acid gastro-oesophageal reflux in idiopathic pulmonary fibrosis. Eur Respir Crit Care Med. 2006; 27:136-142.
18. Raghu G, Weycker D, Edelsberg J, Bradford WZ, Oster G. Incidence and prevalence of idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 2006; 174:810-816.
19. Nadrous HF, Myers JL, Decker PA, Ryu JH. Idiopathic pulmonary fibrosis in patients younger than 50 years. Mayo Clin Proc. 2005; 80:37-40.
20. Nishimura K, Kitaichi M, Izumi T, Nagai S, Kanaoka M, Itoh H. Usual interstitial pneumonia: Histologic correlation with high-resolution CT. Radiology. 1992; 182:337-342.
21. Johkoh T, Muller NL, Cartier Y, Kavanagh PV, Hartman TE, Akira M, Ichikado K, Ando M, Nakamura H. Idiopathic interstitial pneumonia: Diagnostic accuracy of thin-section CT in 129 patients. Radiology. 1999; 211:555-560.
22. Hansell DM, Bankier AA, Macmohan H, McLoud TC, Müller NL, Remy J. Fleischner Society: Glossary of terms for thoracic imaging. Radiology. 2008; 246:697-722.
23. Fell CD, Martinez FJ, Liu LX, Murray S, Han MK, Kazerooni EA, Gross BH, Myers J, Travis WD, Colby TV, Toews GB, Flaherty KR. Clinical predictors of a diagnosis of idiopathic pulmonary fibrosis. Am J Respir Crit Car Med. 2010; 181:832-837.
24. Du Bois RM. An earlier and more confident diagnosis of idiopathic pulmonary fibrosis. Eur Respir Rev. 2012; 21:141-146.
25. Nicholson AG, Addis BJ, Bharucha H, Clelland CA, Corrin B, Gibbs AR, Hasleton PS, Kerr KM, Ibrahim NB, Stewart S,Wallace WA, Wells AU. Inter-observer variation between pathologists in diffuse parenchymal lung disease. Thorax. 2004; 59:500-505.
26. Raghu G, Mageto YN, Lockhart D, Schmidt RA, Wood DE, Godwin JD. The accuracy of the clinical diagnosis of new-onset idiopathic pulmonary fibrosis and other interstitial lung disease: A prospective study. Chest. 1999; 116:1168-1174.
27. Kim DS, Collard HR, King TE Jr. Classification and natural history of the idiopathic interstitial pneumonias. Proc Am Thorac Soc. 2006; 3:285-292.
28. Swensen S, Aughenbaugh G, Myers J. Diffuse lung disease: Diagnostic accuracy of CT in patients undergoing surgical biopsy of the lung. Radiology. 1997; 205:229-234.
29. Hunninghake GW, Zimmerman MB, Schwartz DA, *et al*. Utility of a lung biopsy for the diagnosis of idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 2001; 164:193-196.
30. Flaherty KR, Thwaite EL, Kazerooni EA, Gross BH, Toews GB, Colby TV, Travis WD, Mumford JA, Murray S, Flint A,Lynch JP 3rd, Martinez FJ. Radiological versus histological diagnosis in UIP and NSIP: Survival implications. Thorax. 2003; 58:143-148.
31. Berbescu EA, Katzenstein AL, Snow JL, Zisman DA. Transbronchial biopsy in usual interstitial pneumonia. Chest. 2006; 129:1126-1131.
32. Kinder BW, Brown KK, McCormack FX, Ix JH, Kervitsky A, Schwarz MI, King TE Jr. Serum surfactant protein-A is a strong predictor of early mortality in idiopathic pulmonary fibrosis. Chest. 2009; 135:1557-1563.
33. Satoh H, Kurishima K, Ishikawa H, Ohtsuka M. Increased levels of KL-6 and subsequent mortality in patients with interstitial lung diseases. J Intern Med. 2006; 260:429-434.
34. Prasse A, Probst C, Bargagli E, Zissel G, Toews GB, Flaherty KR, Olschewski M, Rottoli P, Müller-Quernheim J. Serum CC-chemokine ligand 18 concentration predicts outcome in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med. 2009; 179:717-723.
35. Prasse A, Pechkovsky DV, Toews GB, Schäfer M, Eggeling S, Ludwig C, Germann M, Kollert F, Zissel G, Müller-Quernheim J. CCL18 as an indicator of pulmonary fibrotic activity in idiopathic interstitial pneumonias and systemic sclerosis. Arthritis Rheum. 2007; 56:1685-1693.

36. Ishii H, Mukae H, Kadota J, Kaida H, Nagata T, Abe K, Matsukura S, Kohno S. High serum concentrations of surfactant protein A in usual interstitial pneumonia compared with non-specific interstitial pneumonia. Thorax. 2003; 58:52-57.
37. Ohnishi H, Yokoyama A, Kondo K, Hamada H, Abe M, Nishimura K, Hiwada K, Kohno N. Comparative study of KL-6, surfactant protein-A, surfactant protein-D, and monocyte chemoattractant protein-1 as serum markers for interstitial lung diseases. Am J Respir Crit Care Med. 2002; 165:378-381.

(Received March 11, 2015; Revised April 3, 2015; Accepted April 16, 2015)

Review

DOI: 10.5582/irdr.2015.01007

Cardiac manifestations in Behcet's disease

Selami Demirelli[1,*], Husnu Degirmenci[2], Sinan Inci[3], Arif Arisoy[4]

[1] *Selami Demirelli, M.D, Department of Cardiology, Erzurum Education and Research Hospital, Erzurum, Turkey;*
[2] *Husnu Degirmenci, M.D, Department of Cardiology, Erzincan Universty, Erzincan, Turkey;*
[3] *Sinan Inci, M.D, Department of Cardiology, Aksaray State Hospital, Aksaray, Turkey;*
[4] *Arif Arisoy, Department of Cardiology, M.D, Gaziosmanpasa Universty, Medical Faculty, Tokat, Turkey.*

Summary **Behcet's disease (BD) is a chronic inflammatory disorder, with vasculitis underlying the pathophysiology of its multisystemic effects. Venous pathology and thrombotic complications are hallmarks of BD. However, it has been increasingly recognised that cardiac involvement and arterial complications are also important aspects of the course of the disease. Cardiac lesions include pericarditis, endocarditis, intracardiac thrombosis, myocardial infarction, endomyocardial fibrosis, and myocardial aneurysm. Treatment of cardiovascular involvement in BD is largely empirical, and is aimed towards suppressing the vasculitis. The most challenging aspect seems to be the treatment of arterial aneurysms and thromboses due to the associated risk of bleeding. When the prognosis of cardiac involvement in BD is not good, recovery can be achieved through oral anticoagulation, immunosuppressive therapy, and colchicine use. In this review, we summarise the cardiovascular involvement, different manifestations, and treatment of BD.**

Keywords: Behcet's disease, cardiac manifestations, treatment

1. Introduction

Behcet's Disease was named BD or Behcet's syndrome when it was first described by Turkish physician Hulusi Behcet in 1937 (*1*). BD involves widespread vasculitis with recurrent oral and genital ulcers and ocular symptoms as well as musculoskeletal, neurological, cardiac, pulmonary, and gastrointestinal system (GIS) involvement. The underlying pathology is an inflammatory response in the arteries and veins. The prognosis varies from patient to patient. Some patients manifest only skin and mucosal lesions, while others may manifest life-threatening central nervous system (CNS) and GIS involvement and pulmonary artery aneurysms (*2,3*). Cardiac involvement in BD is also referred to as cardiac BD. Cardiac involvement may occur in the form of intracardiac thrombus, endocarditis, myocarditis, pericarditis, endomyocardial fibrosis, coronary arteritis, myocardial infarction, and valvular disease (*4*). Recently, some studies have demonstrated that subclinical left ventricular dysfunction in BD progresses at an early stage (*5,6*). Based on this background, in this review, we summarise the cardiovascular involvement and its manifestations in BD.

Released online in J-STAGE as advance publication March 24, 2015.

**Address correspondence to:*
Dr. Selami Demirelli, Department of Cardiology, Erzurum Education and Research Hospital, Erzurum, Turkey.
E-mail: demirelli23@yahoo.com

2. Epidemiology and pathogenesis

BD mostly affects ethnic groups of Mediterranean and East Asian origin that have historically settled along the historic Silk Road route regions. The prevalence of the disease is 80-370/100,000 in Turkey, whereas it is 13-20/100,000 in Japan, Korea, Iran, Iraq, and Saudi Arabia (*7*). In Europe, the estimated prevalence differs based on latitude, with higher prevalences in the south (*8*). Regional distribution not only affects prevalence, but also affects severity of the disease and organ involvement. For example, it has been reported that GIS involvement in Turkey is 5%, whereas it is 50% in Japan (*9*). The gene frequency of human leukocyte antigen (HLA)-B51, which is thought to play a key role in BD pathogenesis, is higher among those living along the Silk Road route than those living in other parts of the world (*8*). The disease is generally seen in the 3rd and 4th decades; it is rare for the disease to occur during adolescence or after the age of 40. BD is seen more commonly among

Table 1. International Study Group diagnostic criteria for the classification of Behcet's syndrome

- *Recurrent oral ulceration:* minor aphthous, major aphthous, or herpetiform ulceration observed by physician or patient recurring at least three times in a 12-month period
- *Plus two of the following:*
  Recurrent genital ulceration: aphthous ulceration or scarring observed by physician or patient
- *Eye lesions*: anterior uveitis, posterior uveitis, or cells in vitreous on slit lamp examination, or retinal vasculitis observed by ophthalmologist.
- *Skin lesions:* erythema nodosum observed by physician or patient, pseudofolliculitis, papulopustular lesions, or acneiform nodules observed by physician in post-adolescent patients not on corticosteroid treatment.
- *Positive pathergy test:* read by physician at 24–48 h (findings applicable only in absence of other clinical explanations).

the family members of those who contract the disease in adolescence, yet the complications are fewer. The prevalence in males and females differs according to the region. In countries of the Middle East, the disease is seen more among males, whereas it is seen more among females in the countries of North Europe and the United States (*2,10*).

The aetiology of BD has yet to be determined. Viral, bacterial, genetic, environmental, toxic, and immune factors all have been proposed to play roles in its pathogenesis (*11*). The basic histopathological sign of BD is vasculitis in large, medium, and small veins. Lymphocytes, monocytes, and dense neutrophil infiltrations are evident in the lesions. It has been shown that antigenic peptides from strains of streptococcus and *Escherichia coli* can stimulate T cells from BD patients to produce higher quantities of interleukin (IL)-6 and interferon (IFN)-γ, respectively, compared to controls (*12*).

In recent years, there have been numerous findings about BD being an autoinflammatory disease rather than an autoimmune disease. Hypergammaglobulinemia and female dominance, seen in classic autoimmune diseases, are not observed in BD. BD typically has a more severe course in males. The serious complications of vascular disease, CNS disease, and pulmonary disease occur more often in males (*13*).

Today, the most accepted view about BD pathogenesis is an increased response of the innate and acquired immune systems against environmental antigens and autoantigens in addition to a genetic predisposition (*13*). The presence of anti-lymphocyte and anti-cardiolipin antibodies has been reported in BD (*14*), and they are used in the diagnosis of the disease. The fact that BD is seen more in some ethnic groups and has intrafamilial prevalence implicates the role of genetic mechanisms in its pathogenesis. The most discussed genetic indicator is HLA-B51, a subclass of HLA class I. HLA-B51 has been identified as the gene with the strongest association with BD (*7*). The proportion of people who suffer from cardiac involvement is not entirely clear; in previous studies, it was identified in 7-46% of patients (*15,16*).

3. Diagnostic Criteria

Due to the lack of specific clinical and laboratory diagnostic tests, several sets of diagnostic criteria have been proposed. Of these, the criteria of the BD Committee of Japan (1987, revised in 2003) (*17*) and the International Study Group for BD (1990) (*18*) (Table 1) are relatively well validated and used worldwide. Based on the International Study Group for BD criteria, diagnosis requires the presence of recurrent oral ulceration plus at least two other criteria (recurrent genital ulceration, ocular signs, skin lesions, positive pathergy test). The Japanese criteria include major (oral ulcers, skin lesions, including subcutaneous thrombophlebitis and cutaneous hypersensitivity, eye lesions, genital ulcers or scars) and minor criteria (arthritis, gastrointestinal lesions, epididymitis, vascular lesions, central nervous system complications). For a definite diagnosis, the presence of four major criteria is required.

Both sets of criteria lack an appreciation of the importance of various venous and arterial lesions, and thromboembolic complications, frequently reported as part of the initial, oligosymptomatic, or classical overt clinical presentation of BD. Diagnostic uncertainties also include the issue of cardiac involvement. Perhaps a diagnosis of cardio BD in young subjects originating from countries along the Silk Road route should be considered even in cases when the major (classical) criteria, such as oral ulcerations or a positive pathergy test, are absent. In these cases, a detailed analysis of the heart and large vessels structures and functions by echocardiography, magnetic resonance imaging (MRI), computed tomography (CT), and appropriate follow-up will be required.

4. Cardiac involvement in BD

The morphological basis of the systemic manifestations in BD, including cardiovascular involvement, is vasculitis (*19*). More specifically, some pathologists consider perivascular structures as the main target of T lymphocyte-mediated immune reactions, and perivasculitis as an essential part of the vasculopathy in BD (*20,21*). The venous and arterial wall lesions attract cytokinergic and neutrophilic reactions. Activated neutrophils cause destructive effects by excessive production of superoxide anion radicals and lysosomal enzymes. Neutrophilic infiltration and advanced vascular wall destruction with aneurysm formation cause local blood flow abnormalities. Endothelial

dysfunction, release of von Willebrand factor, platelet activation, enhanced thrombin and fibrin generation, antithrombin deficiency, and impaired fibrinolysis close the pathological chain of enhanced thrombocoagulation associated with vasculitis (perivasculitis) in BD (*22,23*).

Cardiovascular involvement in BD is estimated to range from 7% to 46%. In the disease, vasculitis lesions that may affect veins and arteries of all sizes are present. It has been reported that venous involvement is 29%, and arterial involvement varies from 8% to 18% (*24*). Endovascular and perivascular inflammation may result in stenosis, thrombus, and aneurysms. The typical form of arterial involvement manifests as a real or false aneurysm. The frequency of vascular involvement was reportedly 14.3% in 2,319 cases, and was significantly higher in males. The most commonly seen complication was superficial vein thrombosis, followed by deep vein thrombosis (*25*). Blockage of the superior and inferior vena cavae, Budd-Chiari syndrome (hepatic vein thrombosis), and dural sinus thrombosis are other vascular complications (*26*). In arterial involvement, which is less common than venous involvement, the carotid, pulmonary, aorta, iliac, femoral, and popliteal arteries are more commonly affected. Although less frequently, the renal arteries may also be involved. The prognosis of patients with arterial involvement is poor, with death rates of 13.5% in those with arterial lesions, especially when the pulmonary artery and thoracic aorta are involved (*27*).

Coronary aneurysms may be seen during angiography procedures in patients with BD. Some of these aneurysms are asymptomatic, whereas others manifest with acute coronary syndrome (*28,29*). These aneurysms are isolable, and most are evident together with coronary stenosis and are sometimes seen together with several arterial aneurysms (*30,31*). Sinus Valsalva aneurysms and aortitis are the most frequently reported cardiac complications, with BD particularly involving the root of the aorta (*31-33*). Sinus Valsalva aneurysms may be seen alone or with other sinus aneurysms, and may lead to acute or chronic aortic failure.

BD frequently leads to aneurysmal dilation of pulmonary arteries (*34,35*). Such pulmonary arterial involvement shows high mortality and morbidity. Aneurysms caused by the involvement of large proximal branches of pulmonary arteries often occur and are initially symptomatic with hemoptysis. Hemoptysis due to pulmonary arterial aneurysms (PAA) should be differentiated from pulmonary emboli. Anticoagulant and fibrinolytic treatments given, based on a presumption of pulmonary emboli, may result in the death of patients with aneurysms (*36*). The diagnosis is confirmed by pulmonary angiography and CT. In the study by Ma *et al.*(*26*), Bentall operations in BD (aortic valve replacement + ascending aorta replacement) were performed more frequently than in a normal population, and aortic valve separations were seen more frequently among these cases (*37-39*).

Heart involvement in BD may progress as endocarditis, myocarditis, pericarditis, intracardiac thrombosis, endomyocardial fibrosis, and valve diseases. Pericardial involvement has been reported as the most common manifestation of cardiac involvement in some series (*15,40*). The clinical presentation may be acute pericarditis, hemorrhagic pericardial tamponade, constrictive pericarditis, recurrent pericarditis, or even just a small, asymptomatic pericardial effusion (*41,42*). Endomyocardial involvement typically manifests as endomyocardial fibrosis on the right and/or left side of the heart (*15,43-45*).

Interatrial septum aneurysm, mitral valve prolapse, and mitral failure are rarely seen (*46*). Intracardiac thrombus is generally one of the serious cardiac complications, and may be one of the first findings of the disease with pulmonary emboli, or may cause cerebral emboli by passing through the patent foramen ovale. Often, the right ventricle is involved, but it has been demonstrated that the left ventricle can also be involved (*47-49*). In the study by Geri *et al*. (*46*), 807 BD cases aged 30 years on average were examined, and significantly more heart pathologies were found at a rate of 6% compared to the normal population. Of the heart conditions, 38% were pericarditis, 26% were endocarditis, 19% were intracardiac thrombus, 17% were myocardial infarcts, 7% were endomyocardial fibrosis, and 2% were myocardial aneurysm. Among the group with no heart involvement, which was a majority of the patients, BD was detected in the deep and superficial veins at a rate of 59%.

A component of BD is cardiomyopathy, which can be ischemic, non ischemic, or inflammatory in nature. Clinically, it can manifest as systolic or diastolic heart failure, or in a more subtle way, as asymptomatic systolic or diastolic dysfunction (*50,51*). In a study performed in our clinic, 30 BD patients were evaluated by conventional and tissue Doppler parameters. The parameters included peak early diastolic (E) velocity and deceleration time of early diastolic flow (DT), mitral annular early diastolic (Em), and late diastolic (Am) velocities, which were impaired in the early stages of the disease. The strain and strain rate values that showed left ventricular systolic and diastolic function were significantly decreased in the patient group compared to the healthy controls (*5*). In a similar study by Yağmur *et al*. (*6*), meaningful decreases were found in regional and mean longitudinal strain values in the early period in BD cases, but increases were seen in NT proBNP values.

5. Prognosis

BD is a chronic and inflammatory disease and shows relapses and remissions. Among young people and males, its progress may be more severe. As age advances, remissions lengthen and the severity of

relapses reduces. Among factors that affect BD morbidity, ethnicity, geographic, and genetic features have been most emphasised (*52*). The prognosis of mucocutaneous involvement in BD is generally good. Ocular involvement, which starts during the early years of the disease, is the most important cause of morbidity, whereas vascular involvement represents the most important cause of mortality. Neurological involvement is progressive among most patients, and is sometimes fatal. Annual mortality in BD varies between 2% and 4%. The most common causes of mortality are rupture of vascular aneurysms, intestinal ulcer perforations, and MSS involvement. PAAs and Budd-Chiari syndrome are associated with higher mortality rates. Overall survival in BD patients with cardiac involvement is poorer than in those without (*47*).

6. Treatment of BD with Cardiac Involvement

Because the aetiopathogenesis of BD is unknown, there is no specific treatment. Treatment of BD is still based on a low level of evidence (*i.e.*, expert opinions) (*53*). The aim of the treatment is to correct the symptoms, and to prevent the disease from causing permanent organ damage by repressing inflammation. Pharmacological agents used for the treatment of BS include corticosteroids, colchicine, azathioprine, and tumour necrosis factor-α inhibitors, among others. Pericarditis has always been treated with aspirin and immunosuppressive agents (*40*). In the setting of tamponade, emergency pericardiosynthesis may be necessary (*41*). Thrombus management is determined based on the mobility of the thrombus. If the thrombosis seems relatively immobile and is not susceptible to emboli, acetylsalicylic acid, warfarin, corticosteroids, and immunosuppressive agents may be used. However, more mobile thrombuses should be treated with thrombolytic agents (*48,54*). Acute myocardial infarction can be treated with percutaneous coronary intervention or surgical revascularisation (*46*). Myocardial involvement is almost always associated with endomyocarditis. Endomyocardial fibrosis in some cases has been cured with corticosteroids, colchicine, or immunosuppressive agents (*46,55*). High-dose prednisolone and azathioprine can be used, along with the routine treatment of cardiac failure, and in some patients, cardiac function has improved with these medications (*50*). The presence of PAA is associated with higher mortality rates (*56*). Prognosis in these patients becomes better with early diagnosis and intense immunosuppressive treatment (*56,57*). Transcatheter embolectomy has also proven beneficial (*34,35,58*). Oral anticoagulants and antiplatelets are often used to treat thromboembolic complications in BD. However, antithrombotic therapy should be administered cautiously, given the proneness of BD patients to bleedings, particularly from symptomatic or asymptomatic pulmonary aneurysms. Overall survival in BD patients with cardiac involvement is poorer than in those without. Complete remission of cardiac involvement has been associated with the use of immunosuppressants, colchicine, and anticoagulants.

7. Conclusions

BD is a disease with multi-organ involvement and thus shows signs and symptoms in many systems. The main cardiac features of BD include pericarditis, myocardial (diastolic and/or systolic) dysfunction, valvular, coronary (thrombosis, aneurysms, rupture), and intracardiac thrombus. Several cardiac manifestations may coincide in one patient. Cardiologists should always bear in mind the potential threats of symptomatic cardiovascular involvement in BD and consider diagnostic measures (echocardiography, CT, MRI) for its timely detection. The prognosis of cardiac lesions is poorer than that of lesions in others organs involved in BD, but anticoagulation, immunosuppressant agents, and colchicine seem to improve the prognosis of cardiac manifestations in BD.

References

1. Behcet H, Matteson EL. On relapsing, aphthous ulcers of the mouth, eye and genitalia caused by a virus 1937. Clin Exp Rheumatol. 2010; 28(4 Suppl 60):S2-S5.
2. Saadoun D, Wechsler B. Behcet's disease. Orphanet J Rare Dis. 2012; 7:20.
3. Saylan T, Mat C, Fresko I, Melikoğlu M. Behcet's disease in The Middle East. Clin Dermatol. 1999; 17:209-222.
4. Kaklamani VG, Vaiopoulos G, Kaklamanis G. Behcet's disease. Semin Arthritis Rheum. 1998; 27:197-217.
5. Demirelli S, Degirmenci H, Bilen H, Ermis E, Duman H, Arisoy A, Bakirci EM, Ipek E, Askin L. Left ventricular mechanics in Behcet's disease: A speckle tracking echocardiographic study. Bosn J Basic Med Sci. 2014; 14:160-164.
6. Yagmur J, Sener S, Acikgoz N, Cansel M, Ermis N, Karincaoglu Y, Tasolar H, Karakus Y, Pekdemir H, Ozdemir R. Subclinical left ventricular dysfunction in Behcet's disease assessed by two-dimensional speckle tracking echocardiography. Eur J Echocardiogr. 2011; 12:536-541.
7. Zoubloulis CC. Epidemiology of Adamantiades-Behcet's disease. Ann Med Interne. 1999; 150:488-498.
8. Verity DH, Marr JE, Ohno S, Wallace GR, Stanford MR. Behcet's disease, the Silk Road and HLA-B51: Historical and geographical perspectives. Tissue Antigens. 1999; 54:213-220.
9. Yurdakul S, Tuzuner N, Yurdakul I, Hamuryudan V, Yazici H. Gastrointestinal involvement in Behcet's syndrome: A controlled study. Ann Rheum Dis. 1996; 55:208-210.
10. Poon W, Verity DH, Larkin GL, Graham EM, Stanford MR. Behcet's disease in patients of west African and Afro-Caribbean origin. Br J Ophthalmol. 2003; 87:876-878.
11. Onder M, Gürer MA. The multiple faces of Behcet's disease and its aetiological factors. J Eur Acad Dermatol Venereol. 2001; 15:126-136.

12. Hirohata S, Oka H, Mizushima Y. Streptococcal-related antigens stimulate production of IL-6 and interferongamma by T cells from patients with Behcet's disease. Cell Immunol. 1992; 140:410-419.
13. Direskeneli H. Behcet's disease: Infectious aetiology, new autoantigens, and HLA-B51. Ann Rheum Dis. 2001; 60:996-1002.
14. Tokay S, Direskeneli H, Yurdakul S, Akoglu H. Anticardiolipin antibodies in Behcet's disease: A reassessment. Rheumatology. 2001; 40:192-195.
15. Bletry O, Mohattane A, Wechsler B, Beaufils P, Valère P, Petit J, Gourgon R, Grosgogeat Y, Godeau P. Cardiac manifestations of Behcet's disease 12 cases. Presse Med. 1988; 17:2388-2391.
16. O'Duffy JD. Vasculitis in Behcet's disease. Rheumatol Dis Clin North Am. 1990; 16:423-431.
17. Kurokawa SM, Suzuki N. Behcet's disease. Clin Exp Med. 2004; 4:10-20.
18. International Study Group for Behcet's Disease. Criteria for diagnosis of Behcet's disease. Lancet. 1990; 335:1078-1080.
19. Ehrlich GE. Vasculitis in Behcet's disease. Int Rev Immunol. 1997; 14:81-88.
20. Charteris DG, Champ C, Rosenthal AR, Lightman SL. Behcet's disease: Activated T lymphocytes in retinal perivasculitis. Br J Ophthalmol. 1992; 76:499-501.
21. Borhani Haghighi A, Sharifzad HR, Matin S, Rezaee S. The pathological presentations of neuro-Behcet disease: A case report and review of the literature. Neurologist. 2007; 13:209-214.
22. Kosar A, Oztürk M, Haznedaroğlu IC, Karaaslan Y. Hemostatic parameters in Behcet's disease: A reappraisal. Rheumatol Int. 2002; 22:9-15.
23. Kiraz S, Ertenli I, Oztürk MA, Haznedaroğlu IC, Celik I, Calgüneri M. Pathological haemostasis and "prothrombotic state" in Behcet's disease. Thromb Res. 2002; 105:125-133.
24. Gürgün C, Ercan E, Ceyhan C, Yavuzgil O, Zoghi M, Aksu K, Cinar CS, Türkoglu C. Cardiovascular involvement in Behcet's disease. Jpn Heart J. 2002; 43:389-398.
25. Bayraktar Y, Balkanci F, Bayraktar M, Calguneri M. Budd-Chiari syndrome: A common complication of Behcet's disease. Am J Gastroenterol. 1997; 92:858- 862.
26. Ma WG, Zheng J, Zhu JM, Liu YM, Li M, Sun LZ. Aortic regurgitation caused by Behcet's disease: Surgical experience during an 11-year period. J Card Surg. 2012; 27:39-44.
27. Saadoun D, Asli B, Wechsler B, Houman H, Geri G, Desseaux K, Piette JC, Huong du LT, Amoura Z, Salem TB, Cluzel P, Koskas F, Resche-Rigon M, Cacoub P. Long-term outcome of arterial lesions in Behcet disease: A series of 101 patients. Medicine (Baltimore). 2012; 91:18-24.
28. Barcin C, Iyisoy A, Kurşaklioğlu H, Demirtaş E. A giant left main coronary artery aneurysm in a patient with Behcet's disease. Anadolu Kardiyol Derg. 2004; 4:193.
29. Nakahara H, Yamada T, Yokoyama M, Ohshima N, Tanabe S, Nakamura Y, Irie Y, Nakamoto T, Maezawa H, Shimada Y. A huge aneurysm of the left main coronary artery trunk in Behcet's disease. Kyobu Geka. 1988; 41:976-980.
30. Ozeren M, Dogan OV, Dogan S, Yucel E. True and pseudo aneurysms of coronary arteries in a patient with Behcet's disease. Eur J Cardiothorac Surg. 2004; 25:465-467.
31. Oğuzhan A, Gül A, Asik R, Inanc T, Ozdoğru I, Topsakal R, Eryol NK. Multiple vascular aneurysms in Behcet's disease. Anadolu Kardiyol Derg. 2005; 5:154.
32. Lee S, Lee CY, Yoo KJ. Acute myocardial infarction due to an unruptured sinus of Valsalva aneurysm in a patient with Behcet's syndrome. Yonsei Med J. 2007; 48:883-885.
33. Yoshikawa K, Hori H, Fukunaga S, Tayama E, Aoyagi S. Aortic root replacement in Behcet disease. Asian Cardiovasc Thorac Ann. 2007; 15:521-523
34. Kojuri J, Aslani A, Shahrzad S. Large pulmonary artery pseudoaneurysm in a patient with Behcet's disease. J Cardiovasc Med. 2007; 8:1073-1075.
35. Yakut ZI, Odev K. Pulmonary and cardiac involvement in Behcet disease: 3 case reports. Clin Appl Thromb Hemost. 2007; 13:318-322.
36. Seyahi E, Baskurt M, Melikoglu M, Akman C, Olgun DC, Simsek E, Hamuryudan V, Kucukoglu S, Yazici H. The estimated pulmonary artery pressure can be elevated in Behcet's syndrome. Respir Med. 2011; 105:1739-1747.
37. Lee CW, Lee J, Lee WK, Lee CH, Suh CH, Song CH, Park YB, Lee SK, Won YS. Aortic valve involvementin Behcet's disease. A clinical study of 9 patients. Korean J Intern Med. 2002; 17:51-56.
38. Jeong DS, Kim KH, Kim JS, Ahn H. Long-term experience of surgical treatment for aortic regurgitation attributable to Behcet's disease. Ann Thorac Surg. 2009; 87:1775-1782.
39. Uzun O, Akpolat T, Erkan L. Pulmonary vasculitis in behcet disease: A cumulative analysis. Chest. 2005; 127:2243-2253.
40. Godeau P, Wechsler B, Maaouni A, Fagard M, Herreman G. Cardiovascular involvement in Behcet's disease. Ann Dermatol Venereol. 1980; 107:741-747.
41. Kwon CM, Lee SH, Kim JH, Lee KH, Kim HD, Hong YH, Lee CK. A case of Behcet's disease with pericarditis, thrombotic thrombocytopenic purpura, deep vein thrombosis and coronary artery pseudo aneurysm. Korean J Intern Med. 2006; 21:50-56.
42. Okcun B, Baran T, Babalik E, Kücükoglu S. Multichamber masses and constrictive pericarditis in Behcet's disease. Clin Exp Rheumatol. 2003; 21:55.
43. Aouba A, Nebie L, Fabiani JN, Bruneval P, Patri B, De Bandt M. Tricuspid aseptic endocarditis revealing right endomyocardial fibrosis during an unrecognized Behcet's disease. A case report. Presse Med. 2004; 33:1367-1369
44. Soulami S, Nour-Eddine M, Azzouzi L, Bennis A, Chraibi N. Endomyocardial fibrosis of the right heart in Behcet disease. Arch Mal Coeur Vaiss. 1996; 89:917-921.
45. Belmadani K, Dahreddine A, Benyass A, Hda A, Boukili MA, Ohayon V, Archane MI, Pavie A, Gandjbakhch I. Endomyocardial fibrosis in Behcet's disease: A case report of a pseudo-tumoral form. Arch Mal Coeur Vaiss. 2001; 94:282-286.
46. Geri G, Wechsler B, Thi Huong du L, Isnard R, Piette JC, Amoura Z, Resche-Rigon M, Cacoub P, Saadoun D. Spectrum of cardiac lesions in Behcet disease: A series of 52 patients and review of the literature. Medicine (Baltimore). 2012; 91:25-34.
47. Darie C, Knezinsky M, Demolombe-Rague S, Pinède L, Périnetti M, Ninet JF, Ninet J. Cardiac pseudotumor revealing Behcet's disease. Rev Med Interne. 2005; 26:420-424.
48. Fekih M, Fennira S, Ghodbane L, Zaouali RM. Intracardiac thrombosis: Unusual complication of Behcet's

disease. Tunis Med. 2004; 82:785-790.
49. Wechsler B, Du LT, Kieffer E. Cardiovascular manifestations of Behcet's disease. Ann Med Interne. 1999; 150:542-554.
50. Kaatz M, Görnig M, Bocker T, Zouboulis CC, Wollina U. Late manifestation of a fatal Behcet's disease with cardiac involvement and lethal outcome. Dtsch Med Wochenschr. 1998; 123:217-222.
51. Dogan SM, Birdane A, Korkmaz C, Ata N, Timuralp B. Right ventricular thrombus with Behcet's syndrome: Successful treatment with warfarin and immunosuppressive agents. Tex Heart Inst J. 2007; 34:360-362.
52. Akpolat I, Akpolat T, Danaci M, Bariş YS, Kaya N, Kandemir B. Behcet's disease and amyloidosis. Review of the literature. Scand J Rheumatol 1997; 26:477-479.
53. Hatemi G, Silman A, Bang D, *et al*. EULAR recommendations for the management of Behcet disease. Ann Rheum Dis. 2008; 67:1656-1662.
54. Mendes LA, Magraw LL, Aldea GS, Davidoff R. Right ventricular thrombus: An unusual manifestation of Behcet's disease. J Am Soc Echocardiogr. 1994; 7:438-440.
55. Ozatli D, Kav T, Haznedaroglu IC, Büyükaşik Y, Koşar A, Ozcebe O, Dündar SV. Cardiac and great vessel thrombosis in Behcet's disease. Intern Med. 2001; 40:68-72
56. Hamuryudan V, Yurdakul S, Moral F, Numan F, Tüzün H, Tüzüner N, Mat C, Tüzün Y, Ozyazgan Y, Yazïci H. Pulmonary arterial aneurysms in Behcet's syndrome: A report of 24 cases. Br J Rheumatol. 1994; 33:48-51.
57. Hamuryudan V, Er T, Seyahi E, Akman C, Tüzün H, Fresko I, Yurdakul S, Numan F, Yazici H. Pulmonary artery aneurysms in Behcet syndrome. Am J Med. 2004; 117:867-870.
58. San Luis Miranda R, Lázaro Castillo JL, Enciso Gómez R, Arias Monroy LG, Ramírez Reyes HA, León Avila JL, Munayer Calderón J. Right ventricular thrombous and pulmonary artery aneurysms in Behcet's disease. Report of one case. Arch Cardiol Mex. 2007; 77:130-136.

(Received March 1, 2015; Revised March 17, 2015; Accepted March 19, 2015)

Original Article

DOI: 10.5582/irdr.2015.01002

A systematic review of hereditary spherocytosis reported in Chinese biomedical journals from 1978 to 2013 and estimation of the prevalence of the disease using a disease model

Chao Wang[1,2], Yazhou Cui[1], Yan Li[1,2], Xiao Liu[1,2], Jinxiang Han[1,*]

[1] *Key Laboratory for Biotech Drugs of the Ministry of Health, Key Laboratory for Rare Disease of Shandong Province, Shandong Medicinal Biotechnology Center, Shandong Academy of Medical Sciences, Ji'nan, Shandong, China;*
[2] *School of Medicine and Life Sciences, University of Jinan-Shandong Academy of Medical Science, Ji'nan, Shandong, China.*

Summary **Epidemiological information of hereditary spherocytosis in China is slight. This systematic review summarizes the number of cases of hereditary spherocytosis reported in China Biology Medicine disc from 1978 to 2013. In total, 2,043 cases were reported in the past 36 years. We describe its distribution from time and space. We also estimate the literature reported prevalence of hereditary spherocytosis by DisMod-II software, overall prevalence in China was estimated to be: 1.27 cases per 100,000 people in males and 1.49 cases per 100,000 people in females. All results suggest a stronger network of diagnosis and treatment including all levels of hospitals should be created to improve healthcare for hereditary spherocytosis and even other rare diseases in the future, meanwhile we can obtain more useful information for orphan drug designation purposes and make public health decisions regarding such diseases through the use of the burden of disease models.**

Keywords: Hereditary spherocytosis, Bibliographic study, DisMod-II, China

1. Introduction

Hereditary spherocytosis refers to a group of heterogeneous inherited anemias that are characterized by anemia, jaundice, and splenomegaly. The clinical situation of the disease is often most severe during the first year of life, and most newborns have severe anemia, although it is improved after the first year of life. Jaundice is the first clinical situation in newborns, with severe anemia developing during the first few days after birth. Splenomegaly is a frequently observed feature (*1*). The age of onset and severity of the disease vary considerably. Splenectomy is the first choice of treatment in some patients with symptomatic anemia as it eliminates the necessity of repeated transfusions. Recent evidence is emerging that splenectomy may have adverse vascular long term consequences, and whether patients should undergo splenectomy is controversial (*2,3*).

Hereditary spherocytosis is reported worldwide and is the most common hereditary anemia in individuals from northern Europe and northern America (*4-10*). The prevalence of the disease is around 1 in 5,000 to 1 in 2,000 in the above two areas. The prevalence of hereditary spherocytosis in people of other ethnic backgrounds is unknown, but it is much less common. As a kind of rare disease, there is no exact prevalence of hereditary spherocytosis in China. Although China is also actively promoting regulation of rare diseases, the diseases have not been covered by the national health system. However, there are still no official data on "the prevalence of hereditary spherocytosis and the number of cases". So a crucial step is to collect data on hereditary spherocytosis in China. We searched the reported literature or cases in hospitals to obtain the data collection from China.

This study systematically reviewed hereditary spherocytosis reported in the Chinese biomedical literature published over the past 36 years from January 1978 to December 2013 and analyzed the current state and treatment of hereditary spherocytosis in China.

Released online in J-STAGE as advance publication March 31, 2015.

**Address correspondence to:*
Dr. Jinxiang Han, Key Laboratory for Rare Disease Research of Shandong Province, Key Laboratory for Biotech Drugs of the Ministry of Health, Shandong Medical Biotechnological Center, Shandong Academy of Medical Sciences, Ji'nan, Shandong 250062, China.
E-mail: samshjx@sina.com

This study also estimated the rough literature reported prevalence of hereditary spherocytosis in China.

2. Methods

2.1. *Data collection*

2.1.1. *The literature data*

We searched the literature related to hereditary spherocytosis from China Biology Medicine disc (CBMdisc) (*http://www.sinomed.ac.cn/*) that covered articles since January 1978 to December 2013. The CBM database is the largest Chinese biomedical bibliographic database, it includes more than 1,600 kinds of biomedical journals, conference papers and compilations published in Chinese, and includes 7,875,309 literature citations prior to 2013.

Chinese terms and English terms describing hereditary spherocytosis were used to search for publications in the CBM database (*11*). English terms for hereditary spherocytosis were included because most Chinese biomedical publications contain an English abstract. We collected literature related to clinical data, diagnostic information and laboratory data searched in the CBM database. Cases of hereditary spherocytosis with a confirmed diagnosis were included. For each study included, informed consent for publication was obtained from the patient. Patient medical information was carefully compared for series of reports by the same authors or institutions, and redundant cases were excluded.

2.1.2. *Other data*

The World Health Organization (WHO) and Harvard University designed DisMod-II software to calculate the burden of diseases. The software was used to estimate the prevalence of a simple disease model with four possible kinds of data, namely: incidence; mortality of the disease; all other mortality and remission. The model permits calculation of prevalence at a certain age of remission, the mortality and incidence in the age interval (*12*).

The mortality of hereditary spherocytosis was selected from CDC WONDER (Centers for Disease Control and Prevention Wide-Ranging Online Data for Epidemiologic Research) (*http://wonder.cdc.gov/*). The CDC WONDER is the nation's primary data repository for health statistics of the United States, which covers the CDC site data and statistical information service. Through the system, users can retrieve the morbidity and public health data including, scientific research data, survey data, health statistical data, laboratory information and so on (*13*).

All other mortality was collected from the national disease surveillance system death surveillance data sets 2011 which was written by Chinese Center for Disease Control and Prevention that summarizes the national disease surveillance systems (DSPs) 2011 round of population and mortality data (*14*).

The structure of the general population of China was obtained by the sixth national population census of the People's Republic of China, which was published in the National Bureau of Statistics of the People's Republic of China in 2010 (*15*). It is an agency within the State Council of the People's Republic of China charged with the collection and publication of statistics related to the economy, population and society of the People's Republic of China at national and local levels.

2.2. *Statistical analysis*

2.2.1. *The literature data*

SPSS 21.0 was used to input and manage data, it was corrected in accordance with the original data when there was abnormal data or missing values. Descriptive statistical analysis was used for the frequency distribution analysis and description of the data characteristics. Charts were drawn with SPSS and Excel. A map with shades of color reflecting the number of hereditary spherocytosis cases in each city was created with ArcMap 10.0.

2.2.2. *Prevalence of hereditary spherocytosis*

We selected year 2011 for calculation purposes as this was the latest year for which all other mortality data of China were available. The patients of hereditary spherocytosis were selected from the literature data in 2011, then we used the number of patients from 2011 and the structure of the general population of China to calculate incidence. All data were classified by gender and age group because of the requirements of DisMod-II software.

The Multiple Cause of Death database contains mortality and population counts for all USA counties from 1999 to 2013. We extracted deaths from hereditary spherocytosis of 15 years data, then calculated the average mortality which were broken down by gender and age group.

Hereditary spherocytosis is the only congenital hemolytic anemia for which a splenectomy proves consistently beneficial. Recent evidence is emerging that there may be adverse long term vascular consequences of splenectomy, whether patients should undergo splenectomy is controversial and splenectomy does not eradicate birth defects. So remission was assumed to be zero.

Finally, the model was constructed using hereditary spherocytosis mortality data in USA, and remission. All other mortality was also introduced into the model, along with the national disease surveillance system

Table 1. Estimated prevalence and incidence of hereditary spherocytosis by age group, based on incidence in Chinese literature reported and mortality from USA

Age group	Input(×100,000)*				Output(×100,000)			
	Mortality&		Incidence		Prevalence		Incidence	
	Males	Females	Males	Females	Males	Females	Males	Females
< 1	0.0131	0.0069	0.0804	0.1423	0.0179	0.0188	0.0359	0.0377
1-4	0.0000	0.0000	0.0298	0.0320	0.1089	0.1141	0.0377	0.0390
5-14	0.0006	0.0003	0.0457	0.0432	0.3900	0.3947	0.0364	0.0354
15-24	0.0003	0.0010	0.0026	0.0027	0.5715	0.5697	0.0052	0.0049
25-34	0.0010	0.0003	0.0000	0.0020	0.6121	0.5964	0.0045	0.0025
35-44	0.0006	0.0003	0.0016	0.0008	0.6387	0.6117	0.0024	0.0015
45-54	0.0019	0.0012	0.0011	0.0033	0.4583	0.6279	0.0014	0.0037
55-64	0.0004	0.0020	0.0000	0.0000	0.3646	1.2525	0.3828	0.2031
65-74	0.0000	0.0031	0.0000	0.0000	3.1813	5.5225	0.3501	0.7011
75-84	0.0063	0.0052	0.0000	0.0000	11.0010	14.3372	2.5060	0.8380
85+	0.0343	0.0059	0.0000	0.0000	86.3382	22.7439	4.8329	0.6042
Total	0.0011	0.0011	NA	NA	1.2669	1.4862	0.1508	0.0993

* Remission input (assumed to be zero). & Mortality in USA.

death surveillance data sets 2011 of China. Then the ensuing estimates of prevalence were shown by age group and gender.

3. Results

373 reports were searched in CBM database, 297 reports were qualified for inclusion according to our criteria. A total of 2,043 cases of hereditary spherocytosis were reported in the literature. As shown in Figure 1, the number of patients with hereditary spherocytosis reported each year in the CBM database, after 1994 is significantly higher than a few years ago. 71% of the cases were diagnosed at a university hospital, 22% were diagnosed at a municipal hospital, 4% were diagnosed at a provincial hospital, and the remainder (3%) was diagnosed at hospitals on country level or even from smaller communities (Figure 2). The geographic distribution of reported patients is shown in Figure 3. From the map view, more cases were reported in the South and East of China, which have a higher population density and better medical services than other areas. Shandong, Beijing, Liaoning, Hebei and Shanghai ranked among the top 5 provinces or province level municipalities where disorders were reported. In Xizang, Qinghai and Guizhou only a few cases were reported.

In 2011, the number of cases was 114 reported from CBM database, the male: female ratio was 1.04:1. According to the sixth national population census of the people's republic of China, Chinese population totaled 1,332,810,869, with a breakdown by sex of 682,329,104 males and 650,481,765 females. In 2011, overall literature reported prevalence of hereditary spherocytosis in China was estimated to be: 1.27 cases per 100,000 people in males and 1.49 cases per 100,000 people in females (Table 1). From the table we found that the model estimated incidence of hereditary spherocytosis was high before the age of 15 and after

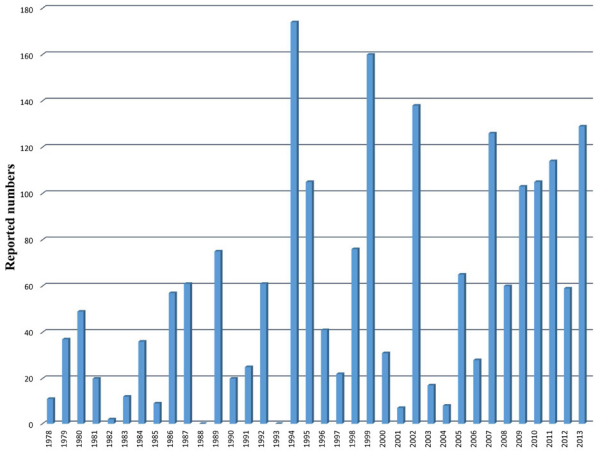


Figure 1. Case numbers of hereditary spherocytosis reported in Chinese biomedical publications from 1978 to 2013. The number of hereditary spherocytosis cases has increased significantly after 1994, and in the last 10 years there are 979 cases that accounted for nearly 1/2 of the reported numbers.

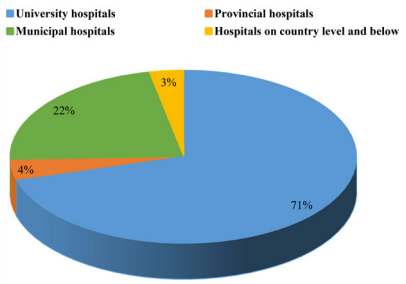


Figure 2. Hospital distribution of reported cases of hereditary spherocytosis in Chinese biomedical Publications. 71% of the cases were diagnosed at a university hospital, 22% at a municipal hospital, 4% at a provincial hospital, and the remainder (3%) was diagnosed at hospitals on country level or even from smaller communities.

the age of 55. We also estimated that China has 8,644 males and 9,667 females hereditary spherocytosis sufferers (Figure 4). The prevalence calculated from national and literature data by the DisMod-II software

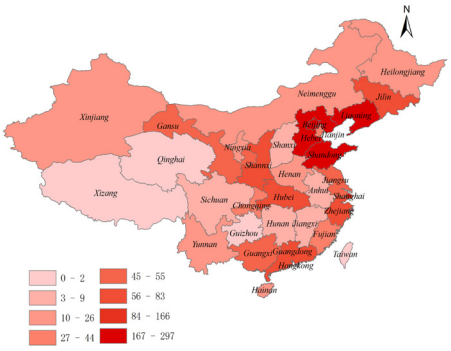


Figure 3. Geographic distribution of reported cases of hereditary spherocytosis in Chinese biomedical publications. More cases were reported in the South and East of China, Shandong, Beijing, Liaoning, Hebei and Shanghai ranked among the top 5 provinces or province level municipalities where cases were reported. Xizang, Qinghai and Guizhou only a few cases were reported.

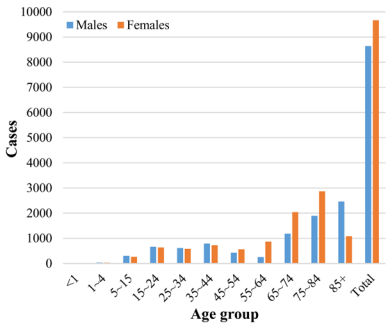


Figure 4. Estimation of hereditary spherocytosis prevalent cases by gender and age groups in China. China was estimated to have 8,644 males and 9,667 females of hereditary spherocytosis sufferers according to the number of hereditary spherocytosis cases reported in Chinese biomedical publications.

is shown in Figure 5. The model estimates an increase with age but with a slower pace, after 65 years cases increase suddenly and at ages > 85 years the estimates reach a maximum.

4. Discussion

Hereditary spherocytosis is a rare disease, there is no cure for hereditary spherocytosis caused by genetic defect, and thus the focus of current management is to limit the severity of the disease. Treatment options include: splenectomy and partial splenectomy, even cholecystectomy. Splenectomy is the most effective way of treatment and will be of benefit in all people with severe as well as some people with moderate hereditary spherocytosis, but is not usually necessary in mild cases. Recent evidence demonstrates that splenectomy for hereditary spherocytosis is safe in the short term, studies showed no deaths and infrequent complications in 1,657 children (*16*). The disadvantages of splenectomy are large trauma and significant immunity decline. Partial splenectomy applied to the young, yields low immunity, and splenectomy might cause serious infection of patients who may relapse

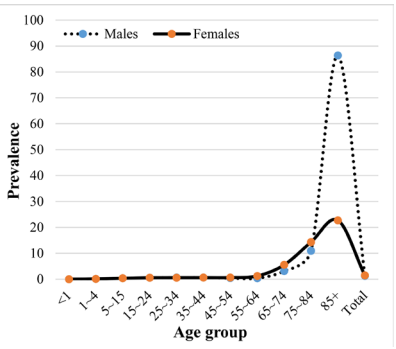


Figure 5. Estimated prevalence of hereditary spherocytosis by gender and age groups in China. Model estimates increase with age but at a slower pace, after 65 years increases suddenly and at ages > 85 years the estimates reach a maximum.

again. Investigators finished a follow-up of 10 years and found that 7.5% cases needed a total splenectomy again (*17*).

From the results of publications in CBM database, the number of hereditary spherocytosis cases increased significantly after 1994, and in the last 10 years there were 979 cases that accounted for nearly 1/2 of the reported numbers. This situation is because general healthcare has improved rapidly and people pay more attention to medical problems caused by rare diseases in China. Cases were more frequently reported around the Bohai area which contains Beijing, Shandong, Liaoning and Hebei. We noted that more university hospitals and blood institutes are in these provinces. Also cases were more reported in large cities instead of areas with a larger population but a relatively lower level of development such as Guizhou and Sichuan Provinces and even Xizang found no reported cases. Country level hospitals and below represent more than 70% of the medical resources in China, but only 3% of the cases of hereditary spherocytosis were diagnosed by these hospitals. There are also some disparities between university hospitals and provincial and municipal hospitals. So, countries should pay more attention to reform health care and improve the level of medical technology of country level hospitals and below. At the same time, a stronger network of diagnosis and treatment including all levels of hospitals should be created to improve healthcare for hereditary spherocytosis and other rare diseases in the future.

Although hereditary spherocytosis is encountered worldwide, its prevalence in other groups (excluding northern European and northern American groups) has not been clearly established. Currently, there is no case registration system for most rare diseases in China, so there is very little information on the epidemiology of hereditary spherocytosis. To our knowledge, a systematic survey of the literature was being performed in order to provide an estimated prevalence of rare diseases or the reported number of published cases in Europe. The prevalence of hereditary spherocytosis was 1/5,000 in Europe which was published on orphanet (the portal for

rare diseases and orphan drugs) in May 2014 (*18*).

DisMod-II was designed as a system to assist in the burden of disease models in the 1990s, and in a few exceptional cases, to estimate disease prevalence (*19-21*). DisMod-II has seven kinds of possible input variables: incidence, remission, case fatality rate, mortality, prevalence, duration and relative risk of death. In general, three input variables are needed to calculate the other four variables and the age of onset. When there are less than three available variables, we can use expert judgment to obtain additional information. The greatest source of error may be from using information that has a lack of data or a small sample size that represents large groups. But the model is fairly robust, it will reasonably allow an estimate of the prevalence, which is comparable with figures reported by published studies. Traditional prevalence studies require more time to collect and calculate data, and only allow input of the number of cases and time or incidence and duration. Nevertheless, DisMod-II permits modelling of variability situations, such as entering different models of remission, incidence and case fatality rate. DisMod-II should be the most effective way to estimate the prevalence of rare diseases.

The model is based on the assumption that incidence and mortality are in a steady state, but it is dependent on the incidence and mortality from the past as well as the present (*22*). The incidence of hereditary spherocytosis was collected and calculated from CBM database reported in 2011, although it would not represent all cases. The mortality of hereditary spherocytosis was selected from CDC WONDER and was an average mortality between 1999 and 2013 in USA. In conclusion, our analysis suggests that previous estimates of prevalence, based on smaller datasets, have underestimated the prevalence of hereditary spherocytosis in China. In the future, we can collect more information from medical record registration systems in hospitals to estimate an accurate prevalence.

5. Conclusion

In conclusion, this systematic review summarized the number of cases and geographic areas of hereditary spherocytosis in China Biology Medicine disc and also estimated the literature reported prevalence of hereditary spherocytosis by DisMod-II software in China. Analyzing the number of cases indicated an imbalance in the distribution of areas and hospitals diagnosis, which suggests a stronger network of diagnosis and treatment including all levels of hospitals that should be created to improve healthcare for hereditary spherocytosis and even other rare diseases in the future. Then we can have more accurate information to estimate prevalence. In addition, through DisMod-II software we can obtain more useful information for orphan drug designation purposes and make public health decisions regarding such diseases.

Acknowledgements

This study was supported by Key Projects in the National Science & Technology Pillar Program during the Twelfth Five-year Plan Period (2013BAI07B01).

References

1. Perrotta S, Gallagher PG, Mohandas N. Hereditary spherocytosis. Lancet. 2008; 372:1411-1426.
2. Shah A. Hemoglobinopathies and other congenital hemolytic anemia. Indian J Med Sci. 2004; 58:490-493.
3. Bolton-Maggs PH, Langer JC, Iolascon A, Tittensor P, King MJ; General Haematology Task Force of the British Committee for Standards in Haematology. Guidelines for the diagnosis and management of hereditary spherocytosis--2011 update. Br J Haematol. 2012; 156:37-49.
4. Bogusławska DM, Heger E, Chorzalska A, Nierzwicka M, Hołojda J, Swiderska A, Straburzyńska A, Paździor G, Langner M, Sikorski AF. Hereditary spherocytosis: Identification of several HS families with ankyrin and band 3 deficiency in a population of southwestern Poland. Ann Hematol. 2004; 83:28-33.
5. Godal HC, Heistø H. High prevalence of increased osmotic fragility of red blood cells among Norwegian blood donors. Scand J Haematol. 1981; 27:30-34.
6. Jensson O, Jónasson JL, Magnússon S. Studies on hereditary spherocytosis in Iceland. Acta Med Scand. 1977; 201:187-195.
7. Eber SW, Pekrun A, Neufeldt A, Schröter W. Prevalence of increased osmotic fragility of erythrocytes in German blood donors: Screening using a modified glycerol lysis test. Ann Hematol. 1992; 64:88-92.
8. Premetis E, Stamoulakatou A, Loukopoulos D. Erythropoiesis: Hereditary spherocytosis in Greece: Collective data on a large number of patients. Hematology. 1999; 4:361-366.
9. Sánchez-López JY, Camacho AL, Magaña MT, Ibarra B, Perea FJ. Red cell membrane protein deficiencies in Mexican patients with hereditary spherocytosis. Blood Cells Mol Dis. 2003; 31:357-359.
10. Ricard MP, Gilsanz F, Millan I. Erythroid membrane protein defects in hereditary spherocytosis. A study of 62 Spanish cases. Haematologica. 2000; 85:994-995.
11. Cui Y, Zhao H, Liu Z, Liu C, Luan J, Zhou X, Han J. A systematic review of genetic skeletal disorders reported in Chinese biomedical journals between 1978 and 2012. Orphanet J Rare Dis. 2012; 7:55.
12. Kruijshaar ME, Barendregt JJ, Hoeymans N. The use of models in the estimation of disease epidemiology. Bull World Health Organ. 2002; 80:622-628.
13. Friede A, Reid JA, Ory HW. CDC WONDER: A comprehensive on-line public health information system of the Centers for Disease Control and Prevention. Am J Public Health. 1993; 83:1289-1294.
14. Chinese Center for Disease Control and Prevention. The national disease detection systems death surveillance data set (Yu Wang, Fu Gao, eds.). People's Medical Publishing House, BJ, China, 2011.
15. National Bureau of Statistics of the People's Republic of China (2010). *http://www.stats.gov.cn/tjsj/pcsj/rkpc/6rp/indexch.htm* (accessed February 1, 2015). (in Chinese)

16. Abdullah F, Zhang Y, Camp M, Rossberg MI, Bathurst MA, Colombani PM, Casella JF, Nabaweesi R, Chang DC. Splenectomy in Hereditary Spherocytosis: Review of 1,657 Patients and Application of the Pediatric Quality Indicators. Pediatr Blood Cancer. 2009; 52:834-837.
17. Bader-Meunier B, Gauthier F, Archambaud F, Cynober T, Miélot F, Dommergues JP, Warszawski J, Mohandas N, Tchernia G. Long-term evaluation of the beneficial effect of subtotal splenectomy for management of hereditary spherocytosis. Blood. 2001; 97:399-403.
18. ORPHANET. Prevalence of rare diseases: Bibliographic data. *http://www.orpha.net/orphacom/cahiers/docs/GB/Prevalence_of_rare_diseases_by_alphabetical_list* (accessed February 1, 2015). (in Chinese).
19. Villaverde-Hueso A, de la Paz MP, Martín-Arribas MC, Sánchez-Valle E, Ramírez-González A, Biairdi P. Prevalence of scleroderma in Spain: An approach for estimating rare disease prevalence using a disease model. Pharmacoepidemiol Drug Saf. 2008; 17:1100-1107.
20. Kruijshaar ME, Barendregt JJ, Van De Poll-Franse LV. Estimating the prevalence of breast cancer using a disease model: Data problems and trends. Popul Health Metr. 2003; 1:5.
21. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: Estimates for the year 2000 and projections for 2030. Diabetes Care. 2004; 27:1047-1053.
22. Murray CJ, Lopez AD. Quantifying disability: Data, methods and results. Bull World Health Organ. 1994; 72:481-494.

(Received February 5, 2015; Revised March 10, 2015; Accepted March 18, 2015)

Original Article

DOI: 10.5582/irdr.2015.01000

A risk factor analysis on disease severity in 47 premature infants with bronchopulmonary dysplasia

Yan Li[1,2], Yazhou Cui[1], Chao Wang[1,2], Xiao Liu[1,2], Jinxiang Han[1,*]

[1] *Key Laboratory for Biotech Drugs of the Ministry of Health, Key Laboratory for Rare Disease of Shandong Province, Shandong Medicinal Biotechnology Center, Shandong Academy of Medical Sciences, Ji'nan, Shandong, China;*
[2] *School of Medicine and Life Sciences, University of Jinan-Shandong Academy of Medical Science, Ji'nan, Shandong, China.*

Summary **Bronchopulmonary Dysplasia (BPD) is a rare chronic lung disease and one of the most difficult complications to treat in premature infants. With the progress at the medical treatment level, an increasing number of BPD premature infants are born, meanwhile, they would be at an increasing risk for numerous complications and rehospitalization because BPD affects many vital organ systems. The pathogenesis of BPD is clearly multifactorial. As the prognosis is closely connected with the severity of BPD, early diagnosis and treatment are of great help to control the development of BPD. This article focuses on risk factors that could influence the severity of BPD in order to provide a reliable basis for early diagnosis, treatment, and better patient assessment.**

Keywords: Bronchopulmonary dysplasia, preterm infants, risk factors

1. Introduction

Bronchopulmonary dysplasia (BPD) is a rare chronic lung disease in premature infants resulting from oxygen and mechanical ventilation that was first described by Northway *et al*. in 1967 (*1*). BPD has become one of the thorniest issues in the neonatal intensive care unit (NICU) and the main cause of chronic respiratory diseases of infants due to auxiliary oxygen for a long time, a high mortality rate, survivors with high reactive airway disease, feeding difficulties and growth retardation. Children with BPD need long-term dependence on oxygen which, easily causes repeated infection of the lung and even leads to physical and intellectual developmental disorders. BPD seriously affects the survival rate and living quality of premature infants and, it brings a heavy burden to the family and society. The reported incidence of BPD was quite different in various studies. Possible reasons were differences in clinical definitions (*2*), demographics of patient populations and management strategies used across studies (*3*). There are about 3,000 ~ 7,000 newborns suffering from BPD in America every year (*4*), however, the morbidity is not clear in China. Many questions about the etiology and pathogenesis of BPD still remain in spite of a mountain of extensive research that has been done which aimed at identifying risk factors of BPD and planning preventative therapies (*5*). The foremost predictor for BPD development is prematurity, yet many other factors may contribute.

In order to study the risk factors which could influence the severity of BPD in premature infants and provide reliable bases for effective prevention and control measures, this research conducted a retrospective analysis concerning the clinical data and inspection results interrelated information of 47 cases. In addition, we also hope to develop better practices in the management of newborns with BPD in the future.

2. Methods

2.1. *Objects*

These cases were selected from the rare diseases database which was established through the pilot project launched by China (*6*). There were 51 BPD infants registered in the database in total from 1st January

Released online in J-STAGE as advance publication March 31, 2015.

**Address correspondence to:*
Dr. Jinxiang Han, Key Laboratory for Rare Disease Research of Shandong Province, Key Laboratory for Biotech Drugs of the Ministry of Health, Shandong Medical Biotechnological Center, Shandong Academy of Medical Sciences, Ji'nan, Shandong 250062, China.
E-mail: samshjx@sina.com

2012 to 31st November 2014. Term infants, cases with congenital anomalies, metabolic or neuromuscular diseases were excluded. Finally, this study consisted of 47 premature infants who were diagnosed with BPD and registered in the database from 2012 to 2014. There were 33 baby boys (70%) and 14 baby girls (30%). All cases corresponded with the BPD diagnosis standard which was formulated by American National Child Health and Human Development Institute (NICHD) in 2000 (*7*). A total of 28 (59%) infants had mild BPD, 13 (28%) moderate BPD, and 6 (13%) with severe BPD. The classification standard of severity was also formulated by NICHD (*7*). Database records included antenatal, natal, and postnatal features together with the laboratory findings of the infants.

2.2. *Data processing and analysis*

SPSS 17.0 was used to input and manage data. Repeated cases have been excluded. Enumeration data was analyzed by chi-square test (Fischer's exact probability test) and measurement data was analyzed by variance analysis. The 95% confidence interval (CI) and p value were acquired by single factor analysis. In this study, we also used the classification of BPD as dependent variable and all factors with significant associations emerging from the univariate analysis as independent variables to conduct an ordinal logistic regression analysis. The difference was statistically significant when $p < 0.05$. Charts were then drawn with SPSS and Excel.

3. Results

3.1. *Antenatal features*

As shown in Table 1, the differences were statistically significant when the pregnant mothers had chorioamnionitis and history of abnormal pregnancy. Repeated abortion history, placenta previa, placental abruption, polyhydramnios, oligohydramnios, edema-hypertension-proteinuria syndrome, *etc* were included in the abnormal pregnancy group.

3.2. *Natal features*

Statistical analysis results showed that the smaller the gestational age and the lower the birth weight are, the higher the severity. The results displayed that multiple births didn't have statistical significance despite three sets of twins that suffered from BPD. In the meantime, gender, delivery mode and intrauterine infection pneumonia didn't influence the severity of BPD according to the value of p. All the natal features are shown in Table 2.

3.3. *Postnatal characteristics*

Abnormal coagulation function, cholestasis, NRDS and acidosis could affect the severity of BPD among postnatal factors while severity of anemia, HIE, disorders of glucose metabolism and hypoproteinemia were not

Table 1. Conditions of mothers during pregnancies

Factors	Mild BPD *n* (%)	Moderate BPD *n* (%)	Severe BPD *n* (%)	*p*
Maternal age				
≤ 30 years old	19 (68)	10 (77)	5 (83)	0.720
31 ~ 42 years old	9 (32)	3 (23)	1 (17)	0.720
Premature rupture of membranes	10 (36)	4 (31)	2 (33)	1.000
Chorioamnionitis	7 (25)	8 (62)	4 (67)	0.025
Prenatal hormone application	4 (14)	4 (31)	2 (33)	0.265
Pregnancy complications	15 (54)	7 (54)	5 (83)	0.446
History of abnormal pregnancy	6 (21)	6 (46)	5 (83)	0.009

Table 2. Conditions after the births of infants

Factors	Mild BPD *n* (%)	Moderate BPD *n* (%)	Severe BPD *n* (%)	*p*
Gestational age				
< 28 weeks	2 (7)	1 (8)	5 (83)	
28 ~ 31 weeks	21 (75)	10 (77)	1 (17)	0.002
32 ~ 35 + 3 weeks	5 (18)	2 (15)	0	
Birth weight				
< 1000 g	0	4 (31)	1 (17)	
1000 ~ 1499 g	21 (75)	6 (46)	3 (50)	0.025
1500 ~ 1999 g	4 (14)	3 (23)	2 (33)	
2000 ~ 2015 g	3 (11)	0	0	
Multiple births	8 (29)	2 (15)	2 (33)	0.626
Baby girl	7 (25)	3 (23)	4 (67)	0.139
Delivery mode				
Eutocia	14 (50)	8 (62)	4 (67)	0.701
Cesarean section	14 (50)	5 (38)	2 (33)	
Intrauterine infection pneumonia	8 (29)	1 (8)	0	0.213

Table 3. Clinical manifestations concerning infants in the duration of hospital stay

Clinical manifestation	Mild BPD *n* (%)	Moderate BPD *n* (%)	Severe BPD *n* (%)	*p*
Anemia				
Mild	5 (18)	5 (39)	0	
Moderate	17 (61)	6 (46)	4 (67)	0.407
Severe	6 (21)	2 (15)	2 (33)	
HIE	23 (82)	10 (77)	6 (100)	0.555
Disorders of glucose metabolism	15 (54)	6 (46)	4 (67)	0.704
Hypoproteinemia	13 (46)	3 (23)	2 (33)	0.394
Abnormal coagulation function	1 (4)	4 (31)	4 (67)	0.001
Cholestasis	3 (11)	4 (31)	4 (67)	0.012
PDA	10 (77)	2 (22)	2 (40)	0.040
Patent foramen ovale	8 (62)	5 (56)	3 (60)	1.000
NRDS	12 (43)	4 (31)	6 (100)	0.012
Acidosis	17 (61)	12 (92)	6 (100)	0.041

HIE, hypoxic-ischemic encephalopathy; PDA, patent ductus arteriosus; NRDS, neonatal respiratory distress syndrome.

Table 4. Ordered logistic regression analysis concerning influence factors of BPD

Clinical manifestation	b*	Standard error	Wald	*p*	OR	95% CI
Chorioamnionitis	3.224	0.983	10.761	0.001	25.128	1.298-5.150
Abnormal pregnancy	1.825	0.912	4.005	0.045	6.203	0.038-3.613
Abnormal coagulation function	2.697	1.078	6.262	0.012	14.835	0.585-4.809
Cholestasis	2.481	1.002	6.136	0.013	11.953	0.518-4.445
Gestational age	3.441	1.410	5.953	0.015	31.218	0.677-6.204
Birth weight	12.523	2.293	29.856	0.0004	274580.567	8.031-17.051

*Regression coefficient.

influence factors with statistical significance (Table 3). There were 13 mild, 9 moderate and 5 severe cases taken electrocardiographic examinations in total. In the echocardiography results, patent ductus arteriosus could influence severity compared with patent foramen ovale after statistical verification.

3.4. *Ordered logistic regression analysis about influence factors of BPD*

Through single factor analysis, 9 factors had statistical significance including: chorioamnionitis, history of abnormal pregnancy, gestational age, birth weight, abnormal coagulation function, cholestasis, NRDS, acidosis and patent ductus arteriosus. In this study, only 27 patients accepted vascular ultrasonography and valid data was too small. Because it didn't meet the conditions of ordered logistic regression analysis, the factor was excluded. By means of ordered logistic regression analysis, chorioamnionitis, history of abnormal pregnancy, abnormal coagulation function, and cholestasis were independent factors which could influence the severity of BPD (Table 4).

4. Discussion

After nearly 5 decades since the first description of BPD by Northway, its epidemiology, clinical presentation and pathogenesis have changed. BPD was a chronic lung disease associated with premature birth and still lacked effective prevention and treatment (*9*). The etiology and pathogenesis were still unclear and most scholars believed that the occurrence and development of BPD were associated with premature birth, inhalation of high concentrations of oxygen, long duration of mechanical ventilation and infection (*10*). Immature lung development at an early gestational age and light weight infants were the most basic factors for the occurrence of BPD (*11-15*). This study also showed BPD was more serious when the gestational age was earlier and birth weight was lower owing to immature lung development and respiratory function.

Our study showed that there is a relationship between the severity of BPD and PDA. The incidence of heart failure, pulmonary edema, *etc*. increased in BPD infants with PDA. It was due to PDA could also significantly increase blood circulation in lung tissue, probability of edema and infection of lung tissue. PDA and BPD may be a cause and effect relationship. Some researchers came to the same conclusion (*16*). Respiratory distress syndrome (RDS) is a common cause of morbidity and mortality related to premature birth and most infants who develop BPD initially suffer from acute RDS (*17*). Infants with RDS may easily have acidosis due to adverse pulmonary ventilation. The present study shows, as we expected, that RDS and acidosis are associated with the severity of BPD.

Chorioamnionitis was an independent risk factor in our study. When pregnant women have chorioamnionitis, the expression levels of the proinflammatory cytokines

increased significantly which could also cause a fetal pulmonary inflammatory reaction or even systemic inflammatory response syndrome. Pulmonary edema, abnormal deposition of fibrin and a decrease of the biological activity of pulmonary surfactant were caused by the pathological process. Inflammatory cytokines in amniotic fluid get into the fetal lung when the mother has chorioamnionitis and caused lung inflammation and injury. The injury continued development after birth and the infants finally developed BPD (*18*).

Our findings indicated that women with a history of abnormal pregnancy (abortion, stillbirth and premature birth) was another risk factor which could influence the severity of BPD. Some scholars believed that pregnant women with an abnormal childbearing history was a potential risk factor for BPD (*19*), but yet no studies showed that abnormal pregnancy was an independent risk factor for the severity of BPD. The endometrium cannot fully repair itself in the short term and a cervical mucus embolism that was erased during the operation could lead bacteria easily into the uterine cavity. A common complication is uterine cervical lesions after abortion and pregnant women are prone to premature birth when they have a subsequent pregnancy because the cervix function is not complete. On the other hand, the endometrial myometrial interface was damaged during the artificial abortion and the placental circulation was disordered which could cause placental insufficiency. Finally, these reasons lead to fetal intrauterine hypoxia, slow growth, premature birth and even stillbirth.

Blood vessels are more vulnerable and coagulation factors are deficient in premature infants. Most premature infants had asphyxia when they were born and the function of the organ and adaptability were weaker compared with term infants which could cause pulmonary, intracranial hemorrhage, *etc*. Therefore, asphyxia could easily cause disorder of blood coagulation function. The development of reticular endothelial structure is imperfect in prematurities. The structure could release tissue factors when the tissue was damaged which was able to initiate the coagulation process. This could further reduce coagulation factors. The situation is more likely to happen when newborns have asphyxia. This is a vicious cycle because hypoxia could accelerate the progress of shock. Therefore, premature infants with abnormal coagulation function and were more commonly applied with mechanical ventilation could further promote the occurrence of BPD. The analytic results in our study also showed abnormal coagulation function was one of the risk factors which could influence the severity of BPD.

We found that cholestasis could influence the severity of BPD, yet no other research has made the same point. Cholestasis means bile acid, bilirubin, *etc* accumulate in the body. A number of studies supported that severe toxicity reactions occurred in the lungs when the concentration of cholic acid increased (*20,21*). Sepulveda found that cholic acid in high concentrations could constrict blood vessels (*22*) which would lead to a decrease in pulmonary blood flow and even pulmonary hypoxia. Some researchers figured that cholic acid may cause lung damage through hindering the synthesis of pulmonary surfactant and cause an inflammatory reaction (*20,23*). These above researches explained how cholestasis could injure the development of the lung which may even cause the occurrence of BPD.

5. Conclusion

Due to an improvement in the survival of ELBW infants, BPD has been increasing over the past several decades. Because BPD arises from multiple pathogenic processes in the preterm lung which probably cause various results, various aspects of care need meticulous assessment. It originates from the interaction of multiple factors that could injure the immature lung, and for exactly that reason, preventions must be developed on the basis of all the factors implicated in its pathogenesis. The more serious the illness is, the worse the prognosis. Severe BPD could even influence the brain or other vital organs in the long term process. Therefore, we can actively take preventive measures to control disease development according to potential risk factors in the clinical performance.

Our data showed that the most relevant risk factors that could influence the severity of BPD were low birth weight, early gestational age, chorioamnionitis, childbearing history, cholestasis and abnormal coagulation function. Comprehending the case history, especially whether the mother had chorioamnionitis or an abnormal pregnancy history, and careful examination were vital. Mothers who have had abortions many times may give birth to severe BPD infants to a greater extent. The birth of premature infants with low birth weight and early gestational age are more likely to develop severe BPD (*24*). Paying close observation to the appearance of cholestasis and abnormal coagulation function could control and prevent the progress of the severity of BPD. It is needed that all NICUs keep making efforts to know better practices, decrease risk factors and contribute to the prevention of BPD.

Acknowledgements

This study was supported by The Key Projects in the National Science & Technology Pillar Program during the Twelfth Five-year Plan Period (2013BAI07B01).

References

1. Northway WH Jr, Rosan RC, Porter DY. Pulmonary disease following respirator therapy of hyaline-membrane disease: Bronchopulmonary dysplasia. N Engl J Med. 1967; 276:357-368.

2. Walsh MC, Yao Q, Gettner P, *et al*. Impact of a physiologic definition on bronchopulmonary dysplasia rates. Pediatrics. 2004; 114:1305-1311.
3. Abman SH, Groothius JR. Pathophysiology and treatment of bronchopulmonary dysplasia. Current issues. Pediatr Clin North Am. 1994; 41:277-315.
4. Monte LF, Silva Filho LV, Miyoshi MH, Rozov T. Bronchopulmonary dysplasia. J Pediatr. 2005; 81:99-110.
5. Eduardo Bancalari. What is the evidence for drug therapy in the prevention and management of bronchopulmonary dysplasia? The newborn lung: Neonatal questions and controversies. 1st ed. Saunders Elsevier, Amsterdam, Netherlands, 2008; pp. 208-232.
6. Cui Y, Zhou X, Han J. China launched a pilot project to improve its rare disease healthcare levels. Orphanet J Rare Dis. 2014; 9:14.
7. Jobe AH, Bancalari E. Bronchopulmonary dysplasia. Am J Respir Crit Care Med. 2001; 163:1723-1729.
8. Wang H, Mu DZ. The treatment and clinical application about drug of bronchopulmonary dysplasia. Chin J Obstet Gynecol Pediatr. 2010; 6:290-295. (in Chinese)
9. Groothuis JR, Makari D. Definition and outpatient management of the very low-birth-weight infant with bronchopulmonary dysplasia. Adv Ther. 2012; 29:297-311.
10. Zhang H, Fu JH, Xue XD. The analysis on high risk factors about bronchopulmonary dysplasia in premature infants with ventilation. Chinese Pediatric Emergency Medicine. 2011; 3:240-242. (in Chinese)
11. Capoluongo E, Ameglio F, Lulli P, Minucci A, Santonocito C, Concolino P, Di Stasio E, Boccacci S, Vendettuoli V, Giuratrabocchetta G, De Cunto A, Tana M, Romagnoli C, Zuppi C, Vento G. Epithelial lining fluid free IGF-I-to-PAPP-A ratio is associated with bronchopulmonary dysplasia in preterm infants. Am J Physiol Endocrinol Metab. 2007; 292:e308-e313.
12. Grisaru-Granovsky S, Reichman B, Lerner-Geva L, Hammerman C, Samueloff A, Schimmel MS; Israel Neonatal Network. Mortality and morbidity in preterm small-for-gestational-age infants: A population-based study. Am J Obstet Gynecol. 2012; 206:150.e1-e7.
13. Bhering CA, Mochdece CC, Moreira ME, Rocco JR, Sant'Anna GM. Bronchopulmonary dysplasia prediction model for 7-day-old infants. J Pediatr. 2007; 83:163-170.
14. Baraldi E, Filippone M. Chronic lung disease after premature birth. N Engl J Med. 2007; 357:1946-1955.
15. Kinsella JP, Greenough A, Abman SH. Bronchopulmonary dysplasia. Lancet. 2006; 367:1421-1431.
16. Stephens BE, Gargus RA, Walden RV, Mance M, Nye J, McKinley L, Tucker R, Vohr BR. Fluid regimens in the first week of life may increase risk of patent ductus arteriosus in extremely low birthweight infants. J Perinatol. 2008; 28:123-128.
17. Gursoy T, Hayran M, Derin H, Ovali F. A clinical scoring system to predict the development of bronchopulmonary dysplasia. Am J Perinatol. 2014. (DOI: 10.1055/s-0034-1393935)
18. Watterberg KL, Demers LM, Scott SM, Murphy S. Chorioamnionitis and early lung inflammation in infants in whom bronchopulmonary dysplasia develops. Pediatrics. 1996; 97:210-215.
19. Walsh MC, Szefler S, Davis J, Allen M, Van Marter L, Abman S, Blackmon L, Jobe A. Summary proceedings from the bronchopulmonary dysplasia group. Pediatrics. 2006; 117:s52-s56.
20. Zecca E, De Luca D, Baroni S, Vento G, Tiberi E, Romagnoli C. Bile acid-induced lung injury in newborn infants: A bronchoalveolar lavage fluid study. Pediatrics. 2008; 121:e146-e149.
21. Why does intrahepatic cholestasis of pregnancy could cause lung injury in newborn? (*http://www.jkb.com.cn/xunyiwenyao/other/2013/0130/21414.html*) (accessed December 20, 2014). (in Chinese)
22. Sepúlveda WH, González C, Cruz MA, Rudolph MI. Vasoconstrictive effect of bile acids on isolated human placental chorionic veins. Eur J Obstet Gynecol Reprod Biol. 1991; 42:211-215.
23. Hills BA, Chen Y, Masters IB, Hills YC. Raised bile acid concentrations in SIDS lungs at necropsy. Arch Dis Child. 1997; 77:120-123.
24. Eriksson L, Haglund B, Odlind V, Altman M, Ewald U, Kieler H. Perinatal conditions related to growth restriction and inflammation are associated with an increased risk of bronchopulmonary dysplasia. Acta Paediatr. 2015; 104:259-263.

(Received January 13, 2015; Revised March 10, 2015; Accepted March 16, 2015)

Original Article

DOI: 10.5582/irdr.2015.01008

Validation of the association of *TCF7L2* and *SLC30A8* gene polymorphisms with post-transplant diabetes mellitus in Asian Indian population

IImran Ali Khan[1,2,3], Parveen Jahan[3], Qurratulain Hasan[1,2], Pragna Rao[4,*]

[1] *Department of Genetics and Molecular medicine, Kamineni Hospitals, Hyderabad, India;*
[2] *Department of Genetics, Vasavi Medical and Research Centre, Khairathabad, Hyderabad, India;*
[3] *Department of Genetics and Biotechnology, Osmania University, Tarnaka, Hyderabad, India;*
[4] *Department of Biochemistry, Kasturba Medical College, Manipal University, Manipal, Karnataka, India.*

Summary **The rs7903146 and rs13266634 polymorphisms in the *TCF7L2* and *SLC30A8* genes, respectively, have been reported to be associated with type 2 diabetes. However, little is known about the association of these polymorphisms with post-transplant diabetes mellitus (PTDM). To study this linkage, we determined a distribution of allele and genotype frequencies in Asian Indians. 42 PTDM and 98 non-PTDM subjects were recruited. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis was performed to detect for rs7903146 and rs13266634 polymorphisms. The clinical details and statistical analysis for PTDM and non-PTDM subjects were recorded. Our results observed higher frequencies of the minor alleles in rs7903146 and rs13266634 polymorphisms in the PTDM group compared to the non-PTDM subjects. The allele frequencies also found to be significantly associated with PTDM (rs7903146: T *vs* C: OR-2.6; (95%CI: 1.2-5.6); p = 0.01; rs13266634: T *vs* C: OR-2.0; (95%CI: 1.1-3.4); p = 0.01). These findings suggest that rs7903146 and rs13266634 polymorphisms are associated with PTDM in the Asian Indian population despite a relatively small study group.**

Keywords: PTDM, non-PTDM, rs7903146, *TCF7L2*, rs13266634, *SLC30A8* and Asian Indians

1. Introduction

New onset diabetes after transplant (NODAT), or Post-transplant diabetes mellitus (PTDM), has become a common complication in renal transplant (RT) subjects (*1*). Tacrolimus (FK506, Tac) and cyclosporine A (CsA) are immunosuppressive drugs commonly used in the treatment after organ transplantation (*2*). Both PTDM and type 2 diabetes mellitus (T2DM) are multifactorial metabolic disorders (*3*). T2DM is a chronic disease, characterized by insulin resistance and impaired insulin secretion from pancreatic β-cells (*4*). PTDM incidence has been increasing dramatically throughout the globe, in parallel to T2DM (*5*). The pathophysiology of PTDM is characterized by a drug-induced deficiency in insulin secretion, rather than deteriorating of insulin resistance over time (*6*).

The appearance of family history of diabetes in predicting PTDM is still query and the relation for the risk factors of PTDM in the general population both from a common mechanistic viewpoint and from a practical viewpoint is unclear (*7*).Genome-wide studies have identified diabetes susceptibility loci on chromosomes 10q25.3 and 8q24.11, where transcription factor 7 like 2 (*TCF7L2*) and solute carrier member 3 zinc transporter member 8 (*SLC30A8*) genes are located (*3*). Earlier studies at the same genetic polymorphisms (*TCF7L2*-rs7903146 and *SLC30A8*-rs13266634) in PTDM subjects (*8,9*). Similar to T2DM, PTDM is a polygenic disease, and inconsistencies in genetic associations have been reported. In many of these studies, hypotheses were originated based on previously reported genetic associations of the predisposition to T2DM in general population, however no clear rationale

**Address correspondence to:*
Dr. Pragna Rao, Department of Biochemistry, Kasturba Medical College, Manipal University, SH 65, Madhav Nagar, Manipal, Karnataka 576104, India.
E-mail: drpragnarao@gmail.com

Table 1. Details of the selection of snips for this study

Gene	region	Chr Region	SNP	rs No.	Forward Primer	Reverse Primer	Band Size	Enzyme
TCF7L2	Intron 3	10q25	C>**T**	rs7903146	ACAATTAGAGAGCTA AGCACTTTTTAGGTA	GTGAAGTGCCC AAGCTTCTC	C: 158/30 bp T: 188 bp	*Rsa*I
SLC30A8	Exon 8	8q24.11	C>**T**	rs13266634	GAAGTTGGAGTCA GAGCAGTC	TGGCCTGTCAAAT TTGGGAA	C: 176/80 bp T: 256 bp	*Hpa*II

was provided for the selection of the investigated genetic variants. Therefore, a well-defined candidate-gene approach is attractive, because it lowers the number of research hypotheses and reduces a chance of finding false genetic associations (*10*). Here we studied the association of rs7903146 (*TCF7L2*) and rs13266634 (*SLC30A8*) gene polymorphisms with the risk of developing PTDM after RT in Asian Indian population.

2. Materials and Methods

2.1. *Patient enrolment*

The study was carried out in Kamineni Hospitals, Hyderabad, India. First, the signed informed consent was obtained from the patients during the recruitment. In total, 140 subjects were recruited. Inclusion and exclusion criteria for selection of subjects were defined previously (*3*). Recruited subjects had undergone renal transplant and on immunosuppressive therapy for more than 3 months. Among them, 42 subjects developed PTDM and 98 were remained as euglycemic subjects, termed non-PTDM as per the American Diabetes Association. Patient information was documented, and the blood was collected to perform polymorphism.

2.2. *DNA and PCR-RFLP analysis*

Two mL of the peripheral blood were collected in tubes containing EDTA. Genomic DNA was extracted using a salting-out technique (*11*). Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay was performed to detect rs7903146 and rs13266634 variations in *TCF7L2* and *SLC30A8* genes. The details of the selected primers, restriction enzymes, and band sizes for digested and undigested PCR products are shown in Table 1. Primers were synthesized by Bio-serve technologies limited, Hyderabad, India. Genotyping was performed using a 25-µL-reaction mix using the Bangalore Genei kit (Bangalore Genei Pvt. Ltd., Bangalore, Karnataka, India), and Applied Biosystems thermal cycler machine (Life Technologies, Carlsbad, CA, USA). *Rsa*I (GT^AC) enzyme was used to detect the rs7903146 polymorphism and *Hpa*II (C^CGG) enzyme for rs13266634 polymorphism. The protocol for PCR-RFLP analysis was described previously (*3*). The digested PCR products were separated by 3.5% agarose gel electrophoresis.

2.3. *Statistical analysis*

Genotype and allele frequencies were calculated using gene-counting method. Assessment of PTDM and non-PTDM parameters with alleles was performed using chi-square and Student's *t* tests. The difference in the occurrence of *TCF7L2*-rs7903146 and *SLC30A8*-rs13266634 genotypes in the PTDM and non-PTDM subjects was evaluated with the chi-square test. The odds ratios and 95% confidence intervals were used in order to determine the relative risks. Data on PTDM and non-PTDM individuals were statistically evaluated using the Openepi software. Hardy-Weinberg Equilibrium (HWE) was tested using the chi-square test with one degree of freedom. $p < 0.05$ was considered statistically significant.

3. Results

3.1. *Clinical investigation*

Current study has enrolled 140 subjects. Among the subjects, 42 had a case of PTDM, and 98 were non-PTDM controls. The clinical details of the PTDM and non-PTDM subjects are presented in Table 2. When we performed the *t*-test between PTDM and non-PTDM subjects, gender, weight, subjects with cyclosporine drugs (*i.e*. CsA, Tac) and along with the dosage were found statistically significant ($p < 0.05$).

3.2. *Genotypes and allele distribution*

Genotype distribution of *TCF7L2* (rs7903146) and *SLC30A8* (rs13266634) polymorphisms in cases and control subjects were in accordance with the HWE. The allele and genotype distribution in the PTDM and non-PTDM subjects are shown in Table 3. The distribution of rs7903146 genotype in PTDM subjects is 31% CC, 42.8% CT and 26.2% TT, while the non-PTDM group consists of 45.9% CC, 33.7% CT and 12.2% TT. The C and T allele frequencies in PTDM are 0.52% and 0.48%, and non-PTDM subjects are 0.75% and 0.25%, respectively. The genotype and allele frequencies were found to be significantly associated when we compared PTDM and non-PTDM subjects (for T *vs* C: OR-2.6; (95%CI: 1.2-5.6); $p = 0.01$ and for CT *vs* CC: OR-2.7; (95%CI: 1.5-4.6); $p = 0.0001$). Simultaneously, the dominant model also positive associated *i.e*. CT + TT *vs* CC: (OR-2.9; (95%CI: 1.3-6.4); $p = 0.006$). The PTDM

Table 2. Baseline characteristics for PTDM and non-PTDM subjects

S. No.	Characteristics	PTDM (n = 42)	non-PTDM (n = 98)	p Value
1	Males/Females	30/12	75/23	0.001
2	Age			
	a) Males (Mean ± S.D.)	39.39 ± 12.12	39.55 ± 10.58	0.27
	b) Females (Mean ± S.D.)	40.01 ± 11.63	39.26 ± 10.87	0.58
3	Weight			
	a) Males (Mean ± S.D.)	62.73±15.81	66.03 ± 12.73	0.08
	b) Females (Mean ± S.D.)	61.71 ± 16.93	65.49 ± 13.68	0.09
4	*a*) On CsA therapy	22	58	0.01
	b) On Tac therapy	20	40	0.02
5	*a*) CsA Dose (mg)	163.88 ± 57.4	201.29 ± 76.86	0.03
	b) Tac Dose (mg)	3.15 ± 1.24	3.11 ± 1.62	0.05
6	*a*) C2 levels (ng/mL) CsA	750 ± 299.03	1024.8 ± 353.42	0.23
	b) Trough levels (ng/mL) Tac	9.55 ± 3.38	8.0 ± 3.32	0.86
7	*a*) C2 levels/dose of CsA	5.24 ± 2.59	5.52 ± 1.97	0.02
	b) Trough levels/dose of Tac	3.62 ± 1.96	2.98 ± 1.49	0.02

Table 3. Genotype and Allele frequencies for rs7903146 and rs13266634 polymorphisms with T2DM and PTDM subjects with their specific control subjects

Genotypes/ Alleles	PTDM cases (n = 42)	Non-PTDM (n = 98)	OR (95% CI)	χ^2	p Value
TCF7L2					
CC	13 (31%)	57 (45.9%)	*Reference*		
CT	18 (42.8%)	33 (33.7%)	2.7 (1.5, 4.6)	13.8	0.0001
TT	11 (26.2%)	8 (12.2%)	3.1 (1.4, 6.6)	8.6	0.003
CT + TT	29 (69%)	41 (54.1%)	2.9 (1.3, 6.4)	7.3	0.006
C	44 (0.52)	147 (0.75)	*Reference*		
T	40 (0.48)	49 (0.25)	2.6 (1.2, 5.6)	6.2	0.01
SLC30A8					
CC	16 (38.1%)	59 (60.2%)	*Reference*		
CT	20 (47.6%)	32 (32.6%)	2.3 (1.0, 5.0)	4.4	0.03
TT	6 (14.3%)	7 (7.1%)	3.1 (0.9, 10.7)	3.5	0.06
CT + TT	26 (61.9%)	39 (39.7%)	2.4 (1.1, 5.1)	5.7	0.01
C	52 (0.62)	150 (0.76)	*Reference*		
T	32 (0.38)	46 (0.23)	2.0 (1.1, 3.4)	6.2	0.01

group consists of 38.1% CC, 47.6% CT and 14.3% TT genotypes in rs13266634 polymorphism, and non-PTDM group consists of 60.2% CC, 32.6% CT and 7.1% TT genotypes. The PTDM group consists of 0.62% of C and 0.38% of T alleles, while the non-PTDM consists of 0.76% C alleles and 0.24% of T alleles. The dominant model, genotype and allele frequencies were significantly associated with PTDM and non-PTDM subjects, *i.e.* for CT + TT *vs* CC: OR-2.4; (95%CI: 1.1-5.1); p = 0.01; T *vs* C: OR-2.0; (95%CI: 1.1-3.4); p = 0.01 and for CT *vs* CC: OR-2.3; (95%CI: 1.0-5.0); p = 0.03).

3.3. *Distribution between calcineurin inhibitors dosage in PTDM and non-PTDM subjects*

The genotype distribution was studied in PTDM and non-PTDM subjects who received treatments with CsA and Tac. Calculations of genotype and allele frequencies were performed using odds ratio and 95% confidence intervals, and are shown in Table 4. Treatment with CsA was significantly associated with rs7903146 genotypes in PTDM and non-PTDM subjects (T *vs* C: OR-3.3 (95%CI: 1.5-6.9); p = 0.001 and CT + TT *vs* CC: OR-4.3 (95%CI: 1.4-12.8); p = 0.005). However, none of the rs7903146 genotypes were significantly associated with Tac treatment in rs7903146 polymorphism (T *vs* C: OR-2.1 (95%CI: 0.9-4.6); p = 0.05 and CT + TT *vs* CC: OR-2.0 (95%CI: 0.6-6.2); p = 0.20). There was no association of treatment with CsA or Tac and rs13266634 polymorphism in both PTDM and non-PTDM subjects ($p > 0.05$).

4. Discussion

Currently, India is the second populous country in the World, after China. A high prevalence of endogamy and relatively low admixture distinguishes Asian Indians from most of other populations presently used in genetic studies (*12*). Life style and pharmacological intervention are important in prevention of diabetes in high-risk patients. Family history and genetic information are the practical tools in the identification of high-risk subjects (*13*). In the present study, we inspected the positive association of *TCF7L2* (rs7903146) and *SLC30A8* (rs13266634) polymorphisms with PTDM in Asian Indians. Allele and genotype frequencies were

Table 4. Genotype distribution between CsA and Tac dosage in PTDM and non-PTDM subjects

Genotypes	CsA (n = 80)				Tac (n = 60)			
	PTDM (n = 22)	non-PTDM (n = 58)	OR (95% CI)	p Value	PTDM (n = 20)	non-PTDM (n = 40)	OR (95% CI)	p Value
rs7903146								
CC	6 (27.3%)	36 (62%)	*Reference*		7 (35%)	21 (52.5%)	*Reference*	
CT	11 (50%)	19 (32.8%)	3.4 (1.1-10.8)	0.02	7 (35%)	14 (35%)	1.5 (0.4-5.2)	0.52
TT	5 (22.7%)	3 (5.1%)	10 (1.8-53.2)	0.002	6 (30%)	5 (12.5%)	3.6 (0.8-15.5)	0.07
CT + TT	16 (72.7%)	22 (37.9%)	4.3 (1.4-12.8)	0.005	13 (65%)	19 (47.5%)	2.0 (0.6-6.2)	0.20
C	23 (0.52)	91 (0.78)	*Reference*		21 (52.5)	56 (0.70)	*Reference*	
T	21 (0.48)	25 (0.24)	3.3 (1.5-6.9)	0.001	19 (47.5)	24 (0.30)	2.1 (0.9-4.6)	0.05
rs13266634								
CC	9 (40.9%)	35 (60.3%)	*Reference*		7 (35%)	24 (60%)	*Reference*	
CT	9 (40.9%)	19 (32.8%)	2.3 (1.0, 5.0)	0.26	11 (55%)	13 (32.5%)	2.9 (0.9-9.2)	0.06
TT	4 (18.2%)	4 (6.9%)	3.1 (0.9, 10.7)	0.07	2 (10%)	3 (7.5%)	2.2 (0.3-16.5)	0.40
CT + TT	13 (59.1%)	23 (39.7%)	2.4 (1.1, 5.1)	0.11	13 (65%)	16 (40%)	2.7 (0.9-8.4)	0.06
C	27 (0.61)	89 (0.77)	*Reference*		25 (62.5)	61 (0.76)	*Reference*	
T	17 (0.39)	27 (0.23)	2.0 (1.1, 3.4)	0.05	15 (37.5)	19 (0.24)	1.9 (0.8-4.3)	0.11

significantly associated with both polymorphisms ($p < 0.05$). This is a preliminary study, and our data is in accordance with earlier studies in other ethnicities looking at rs7903146 polymorphism (*8,14*), but not the rs13266634 polymorphism in PTDM subjects (*9*). The molecular mechanism underlying the progression of diabetes in PTDM is not fully understood (*15*). There are different forms of diabetes, such as type 1 diabetes mellitus (latent autoimmune diabetes of adults), T2DM, gestational diabetes, PTDM/NODAT, maturity onset diabetes of the young. These types have been ascribed to genetic defects in β-cell function or insulin action, endocrinopathies, diseases of the exocrine pancreas, and drug- or chemical induced and transient diabetes. However, there is no consensus on molecular diagnosis and screening in identification of diabetes patients. Current diagnosis and screening are based on biochemical analysis of coagulated blood serum (*4*). The results of the present study reveal that there is an association between *TCF7L2* and *SLC30A8* gene polymorphisms in PTDM in Asian Indians. In the previous study we have shown this association with PTDM compared to normal controls. We found a significant association of PTDM with both the alleles and genotypes ($p < 0.05$) (*14*). Present study was carried out comparing PTDM and non-PTDM subjects.

Several studies have observed at T2DM susceptibility loci. For instance, the associations were established between PTDM and genetic mutations (*16*) and T2DM genetic polymorphisms, such as *PPARG*, *POR*, *IGFBP2*, *ADIPONECTIN*, *KCNQ1*, *CALPAIN*, and *INTERLEUKIN* genes (*8,10,17-20*). Few genetic polymorphisms appeared as negative association, such as *SLC30A8*, *IGFBP2*, *HHEX*, *CDKN2A/B*, *PPARG*, *KCNJ11*, and *TCF7L2* (*9,21*). Our studies showed positive association towards rs7903146 and rs13266634 polymorphisms and not in accordance with the prior studies carried out in PTDM subjects in different ethnicities (*9,15,21*). The associations of diabetic diseases with rs7903146 and rs13266634 polymorphisms have been carried in Indian population (*14,22-29*). Our results showed the positive association between rs7903146 polymorphism and T2DM (submitted for publication) and GDM (unpublished). We also showed a varying association of rs13266634 polymorphism with T2DM (submitted for publication), and a significant association with GDM (unpublished). Meta-Analysis studies showed positive and negative associations of rs7903146 and rs13266634 polymorphisms and T2DM (*13,22,30*).

Earlier genetic studies have suggested the involvement of multiple genes in the pathogenesis of T2DM (*13*). GWAS studies identified hundred, thousands to million single nucleotide polymorphisms (SNPs) in various genes. Among them, *TCF7L2* and *SLC30A8* have been identified on 10q25.3 and 8q24.11 chromosomes containing rs7903146 and rs13266634 polymorphisms in intron 3 and exon 8 regions, respectively. These polymorphisms in the genes have been shown to be associated with impaired β-cell function (*31-32*). Sequence variations in introns or exons may affect the correct processing of the mRNA by disrupting the splice site or altering the secondary structure of the mRNA. Therefore, intronic variations can cause a blockage of translation and decrease in the protein expression levels (*33*). The initial GWAS reports discovered the diabetic genes in T2DM patients in Iceland and French populations (*34,35*). *TCF7L2* is a key component of the Wnt signaling pathway involved in the regulation of pancreatic β-cell proliferation, differentiation and insulin secretion, which regulates glucose metabolism, and rs7903146 polymorphism may operate *via* impaired glucagon-like peptide 1 secretion, stimulated more by fat than by carbohydrate ingestion. Association between *TCF7L2* variants and T2DM is supported by several prospective mechanisms, such

as decreased β-cell mass, liver insulin resistance, and altered chromatin state in 'T' allele carriers (*22,36*). The rs13266634 is an amino acid substitution (Tryptophan-Arginine) polymorphism most commonly associated with T2DM. The C325T missense variant is present in *SLC30A8* gene, product of which belongs to zinc transporter family involved in Insulin secretion (*25*). It plays a major role in transporting zinc from the cytoplasm to intracellular insulin-containing vesicles for insulin maturation, storage, and secretion from pancreatic β-cells (*37*). Zinc plays an important role in all processes of insulin trafficking, *i.e*. synthesis, storage, and secretion (*14*). Previous studies showed no association of rs13266634 polymorphism with PTDM in different ethnicities (*9,15,21*), as well with T2DM in Indian population (*25,28*). Chauhan *et al*., (*27*) and our earlier study (*14*) showed a positive association of rs13266634 polymorphism with PTDM. These results are consisted with our current finding in PTDM and non-PTDM. The results from the same population may vary due to the small sample size. Therefore, we calculated the power value of each analysis, and found it to be 76% and 78%. Thus, we are confident that our results are reliable. Transplant recipients on immunosuppressive therapy are at a particularly high risk of developing PTDM, which is a major adverse effect of immunosuppressive drugs used in the RT patients. The diabetogenicity of calcineurin inhibitors such as CsA and Tac have enabled this treatment to obtain its current place to demonstrate in humans to be mediated through suppression of pancreatic insulin secretion (*38*). These drugs are used to prevent rejection in patients with organ transplant. The role of immunosuppressive agents is to suppress the synthesis of DNA containing the blueprint of genetic information.

The major limitation of our current study was a lack of the information about glucose values, clinical data, selected the single SNP from each gene and small sample size. In conclusion, diabetes risk alleles in *TCF7L2* (rs7903146) and *SLC30A8* (rs13266634) are associated with PTDM subjects in Asian Indians, despite a relatively small study group. PTDM is an important health issue, and it is important to find biomarkers that can predict the risk of developing PTDM (*16*). Our findings could be used in finding and treatment of the high-risk renal transplant subjects with immunosuppressive drugs. Future experimental and mechanistic studies with a larger sample size, accurate clinical data in different ethnicities are warranted.

Acknowledgements

Our team is thankful to Kiran Kumar Vattam for providing the genomic DNA for PTDM and non-PTDM subjects. Our team is grateful to ICMR for providing the project funding and SRF to Imran Ali Khan Mohammed (Sanction no. 5-3-8-39-2007; RHN).

References

1. Chen QJ, Li J, Zuo SR, Zhang YP, Jia SJ, Yuan H, Liu SK, Cheng K, Ming YZ, Zuo XC, Luo AJ, Xie WZ, Cai JJ, Yang M, Wang JL. Tacrolimus decreases insulin sensitivity without reducing fasting insulin concentration: A 2-year follow-up study in kidney transplant recipients. Ren Fail. 2015; 1-6. (doi:10.3109/0886022X.2015.1007833).
2. Gaynor JJ, Ciancio G, Guerra G, *et al*. Multivariable risk of developing new onset diabetes after transplant - results from a single-center study of 481 adult, primary kidney transplant recipients. Clin Transplant. 2015; (doi: 10.1111/ctr.12510).
3. Khan IA, Mukkavali KK, Jahan P, Hasan Q, Rao P. Correlation between *KCNQ1* and *KCNJ11* gene polymorphisms and type 2 and post-transplant diabetes mellitus in the Asian Indian population. Genes & Diseases. 2015; (10.1016/j.gendis.2015.02.009).
4. Khan IA, Movva S, Shaik NA, Chava S, Jahan P, Mukkavali KK, Kamineni V, Hasan Q, Rao P. Investigation of Calpain 10 (rs2975760) gene polymorphism in Asian Indians with Gestational Diabetes Mellitus. Meta Gene. 2014; 2:299-306.
5. Suarez O, Pardo M, Gonzalez S, Escobar-Serna DP, Castaneda DA, Rodriguez D, Osorio JC, Lozano E. Diabetes mellitus and renal transplantation in adults: Is there enough evidence for diagnosis, treatment, and prevention of new-onset diabetes after renal transplantation? Transplant Proc. 2014; 46:3015-3020.
6. Werzowa J, Saemann M, Haidinger M, Krebs M, Hecking M. Antidiabetic therapy in post kidney transplantation diabetes mellitus. Transplant Rev (Orlando). 2015. pii: S0955-470X(15)00002-6. (doi: 10.1016/j.trre.2015.01.001).
7. Ghisdal L, Van Laecke S, Abramowicz MJ, Vanholder R, Abramowicz D. New-onset diabetes after renal transplantation: Risk assessment and management. Diabetes Care. 2012; 35:181-188.
8. Kurzawski M, Dziewanowski K, Kędzierska K, Wajda A, Lapczuk J, Droździk M. Association of transcription factor 7-like 2 (*TCF7L2*) gene polymorphism with posttransplant diabetes mellitus in kidney transplant patients medicated with tacrolimus. Pharmacol Rep. 2011; 63:826-833.
9. Kurzawski M, Dziewanowski K, Lapczuk J, Wajda A, Droździk M. Analysis of common type 2 diabetes mellitus genetic risk factors in new-onset diabetes after transplantation in kidney transplant patients medicated with tacrolimus. Eur J Clin Pharmacol. 2012; 68:1587-1594.
10. Elens L, Sombogaard F, Hesselink DA, Van Schaika RH, Van Gelderb T. Single-nucleotide polymorphisms in P450 oxidoreductase and peroxisome proliferator-activated receptor-α are associated with the development of new-onset diabetes after transplantation in kidney transplant recipients treated with tacrolimus. Pharmacogenet Genomics. 2013; 23:649-657.
11. Khan IA, Kamineni V, Poornima S, Jahan P, Hasan Q, Rao P. Tumor necrosis factor alpha promoter polymorphism studies in pregnant women. Journal of Reproductive Health and Medicine. 2015; 1:18-22.
12. Pemberton TJ, Mehta NU, Witonsky D, Di Rienzo A, Allayee H, Conti DV, Patel PI. Prevalence of common disease-associated variants in Asian Indians. BMC Genet. 2008; 9:13.
13. Chang YC, Liu PH, Yu YH, Kuo SS, Chang TJ, Jiang

YD, Nong JY, Hwang JJ, Chuang LM. Validation of type 2 diabetes risk variants identified by genome-wide association studies in Han Chinese population: A replication study and meta-analysis. PLoS One. 2014; 9:e95045.
14. Vattam KK, Movva S, Khan IA, Upendram P, Mukkavali KK, Rao P, Hasan Q. Importance of Gene Polymorphisms in Renal Transplant Patients to Prevent Post Transplant Diabetes. J Diabetes Metab. 2014; 5:12.
15. Kang ES, Kim MS, Kim YS, Kim CH, Han SJ, Chun SW, Hur KY, Nam CM, Ahn CW, Cha BS, Kim SI, Lee HC. A polymorphism in the zinc transporter gene *SLC30A8* confers resistance against posttransplantation diabetes mellitus in renal allograft recipients. Diabetes. 2008; 57:1043-1047.
16. Tavira B, Gómez J, Díaz-Corte C, Llobet L, Ruiz-Pesini E, Ortega F, Coto E. Mitochondrial DNA haplogroups and risk of new-onset diabetes among tacrolimus-treated renal transplanted patients. Gene. 2014; 538:195-198.
17. Nicoletto BB, Souza GC, Fonseca NK, Centenaro A, Manfro RC, Canani LH, Gonçalves LF. Association between 276G/T adiponectin gene polymorphism and new-onset diabetes after kidney transplantation. Transplantation. 2013; 96:1059-1064.
18. Kim YG, Ihm CG, Lee TW, Lee SH, Jeong KH, Moon JY, Chung JH, Kim SK, Kim YH. Association of genetic polymorphisms of interleukins with new-onset diabetes after transplantation in renal transplantation. Transplantation. 2012; 93:900-907.
19. Tavira B, Coto E, Díaz-Corte C, Ortega F, Arias M, Torres A, Díaz JM, Selgas R, López-Larrea C, Campistol JM, Ruiz-Ortega M, Alvarez V; Pharmacogenetics of Tacrolimus REDINREN Study Group. KCNQ1 gene variants and risk of new-onset diabetes in tacrolimus-treated renal-transplanted patients. Clin Transplant. 2011; 25:E284-E291.
20. Bamoulid J, Courivaud C, Deschamps M, Mercier P, Ferrand C, Penfornis A, Tiberghien P, Chalopin JM, Saas P, Ducloux D. IL-6 promoter polymorphism -174 is associated with new-onset diabetes after transplantation. J Am Soc Nephrol. 2006; 17:2333-2340.
21. Chakkera HA, Hanson RL, Raza SM, DiStefano JK, Millis MP, Heilman RL, Mulligan DC, Reddy KS, Mazur MJ, Hamawi K, Moss AA, Mekeel KL, Cerhan JR. Pilot study: Association of traditional and genetic risk factors and new-onset diabetes mellitus following kidney transplantation. Transplant Proc. 2009; 41:4172-4177.
22. Hussain H, Ramachandran V, Ravi S, Sajan T, Ehambaram K, Gurramkonda VB, Ramanathan G, Bhaskar LV. *TCF7L2* rs7903146 polymorphism and diabetic nephropathy association is not independent of type 2 diabetes--a study in a south Indian population and meta-analysis. Endokrynol Pol. 2014; 65:298-305.
23. Singh K, Agrawal NK, Gupta SK, Singh K. Association of variant rs7903146 (C/T) single nucleotide polymorphism of *TCF7L2* gene with impairment in wound healing among north Indian type 2 diabetes population: A case-control study. Int J Low Extrem Wounds. 2013; 12:310-315.
24. Uma Jyothi K, Jayaraj M, Subburaj KS, Prasad KJ, Kumuda I, Lakshmi V, Reddy BM. Association of *TCF7L2* gene polymorphisms with T2DM in the population of Hyderabad, India. PLoS One. 2013; 8:e60212.
25. Kommoju UJ, Maruda J, Kadarkarai S, Irgam K, Kotla JP, Velaga L, Mohan Reddy B. No detectable association of *IGF2BP2* and *SLC30A8* genes with type 2 diabetes in the population of Hyderabad, India. Meta Gene. 2013; 1:15-23.
26. Ali S, Chopra R, Manvati S, Singh YP, Kaul N, Behura A, Mahajan A, Sehajpal P, Gupta S, Dhar MK, Chainy GB, Bhanwer AS, Sharma S, Bamezai RN. Replication of type 2 diabetes candidate genes variations in three geographically unrelated Indian population groups. PLoS One. 2013; 8:e58881.
27. Chauhan G, Spurgeon CJ, Tabassum R, Bhaskar S, Kulkarni SR, Mahajan A, Chavali S, Kumar MV, Prakash S, Dwivedi OP, Ghosh S, Yajnik CS, Tandon N, Bharadwaj D, Chandak GR. Impact of common variants of *PPARG*, *KCNJ11*, *TCF7L2*, *SLC30A8*, *HHEX*, *CDKN2A*, *IGF2BP2*, and *CDKAL1* on the risk of type 2 diabetes in 5,164 Indians. Diabetes. 2010; 59:2068-2074.
28. Sanghera DK, Ortega L, Han S, Singh J, Ralhan SK, Wander GS, Mehra NK, Mulvihill JJ, Ferrell RE, Nath SK, Kamboh MI. Impact of nine common type 2 diabetes risk polymorphisms in Asian Indian Sikhs: PPARG2 (Pro12Ala), IGF2BP2, *TCF7L2* and FTO variants confer a significant risk. BMC Med Genet. 2008; 9:59.
29. Chandak GR, Janipalli CS, Bhaskar S, Kulkarni SR, Mohankrishna P, Hattersley AT, Frayling TM, Yajnik CS. Common variants in the *TCF7L2* gene are strongly associated with type 2 diabetes mellitus in the Indian population. Diabetologia. 2007; 50:63-67.
30. Tong Y, Lin Y, Zhang Y, Yang J, Zhang Y, Liu H, Zhang B. Association between *TCF7L2* gene polymorphisms and susceptibility to type 2 diabetes mellitus: A large Human Genome Epidemiology (HuGE) review and meta-analysis. BMC Med Genet. 2009; 10:15.
31. Manolio TA, Collins FS, Cox NJ, *et al*. Finding the missing heritability of complex diseases. Nature. 2009; 461:747-753.
32. Kirchhoff K, Machicao F, Haupt A, Schäfer SA, Tschritter O, Staiger H, Stefan N, Häring HU, Fritsche A. Polymorphisms in the *TCF7L2*, *CDKAL1* and *SLC30A8* genes are associated with impaired proinsulin conversion. Diabetologia. 2008; 51:597-601.
33. Tokat B, Kurt O, Bugra Z, Ozturk O, Yilmaz-Aydogan H. Investigation of the monocyte diapedesis-related LFA-1 and JAM-A gene variants in Turkish coronary heart disease patients. Meta Gene. 2013; 2:1-10.
34. Grant SF, Thorleifsson G, Reynisdottir I, *et al*. Variant of transcription factor 7-like 2 (*TCF7L2*) gene confers risk of type 2 diabetes. Nat Genet. 2006; 38:320-323.
35. Sladek R, Rocheleau G, Rung J, *et al*. A genome-wide association study identifies novel risk loci for type 2 diabetes. Nature. 2007; 445:881-885.
36. Franceschini N, Shara NM, Wang H, Voruganti VS, Laston S, Haack K, Lee ET, Best LG, Maccluer JW, Cochran BJ, Dyer TD, Howard BV, Cole SA, North KE, Umans JG. The association of genetic variants of type 2 diabetes with kidney function. Kidney Int. 2012; 82:220-225.
37. Zhang X, Guan SL, Wang ZQ, You Y, Sun SL, Hui L, Miao LN, Yu Y, Kosten TR, Zhang XY. No association between the type 2 diabetes mellitus susceptibility gene, *SLC30A8* and schizophrenia in a Chinese population. Hum Psychopharmacol. 2012; 27:392-396.
38. Penfornis A, Kury-Paulin S. Immunosuppressive drug-induced diabetes. Diabetes Metab. 2006; 32:539-546.

(Received March 1, 2015; Revised April 11, 2015; Accepted April 16, 2015)

Original Article

DOI: 10.5582/irdr.2015.01011

Meconium peritonitis: Prenatal diagnosis of a rare entity and postnatal management

Keiichi Uchida*, Yuhki Koike, Kohei Matsushita, Yuka Nagano, Kiyoshi Hashimoto, Kohei Otake, Mikihiro Inoue, Masato Kusunoki

Department of Gastrointestinal and Pediatric Surgery, Mie University Graduate School of Medicine, Mie, Japan

Summary **The aims of this study were to review our therapy and outcome for meconium peritonitis (MP) patients, and to clarify predictors of postoperative morbidity and mortality. We retrospectively reviewed a total 15 patients with MP who received surgical intervention at our institute from December 1990 to November 2012. Diagnosis of MP was confirmed by operative findings. We analyzed the relationship between outcome and patients' factors including patients' characteristics, prenatal diagnosis, type of MP, general condition, and surgical procedure. There was no relationship between outcome and the following factors: gender, gestational age, body weight at birth, delivery type, Apgar score, prenatal diagnosis, types and causes of MP, and surgical procedure. However, the preoperative presence of circulation deficiency and serum CRP values were statistically significant predictors of outcome in our MP patients. Prenatal diagnosis is essential for the first step of perinatal therapy for MP. Surgical strategy should be selected according to the information of prenatal diagnosis. Early surgical procedures to reduce systemic and abdominal inflammation just after birth may improve the outcome of severe MP cases.**

Keywords: Meconium peritonitis, prenatal diagnosis, surgery

1. Introduction

Meconium peritonitis (MP) is an aseptic chemical peritonitis, which results from perforation of the gut in utero (*1*). Possible causes and pathogenesis of MP include ischemia in the mesentery, volvulus, intestinal atresia, meconium plugs, internal hernia, Hirschsprung's disease, colon atresia, torsion of a fallopian tube cyst, and cystic fibrosis (*1-7*). It can have a wide range of presentations and is classified into 3 types as follows: generalized, cystic, and fibroadhesive types (*1*). Fifty years ago, the mortality rate of MP was reported as approximately 70% (*8*). Recently, the survival rate for MP increased to over 90% (*9*). This improvement is the result of an advance in fetal diagnostic techniques and proper management including surgical procedures and intensive care after birth (*2,10,11*).

All cases of MP have the same etiology; perforation of the intestine in utero and intraperitoneal inflammation by subsequent spillage of meconium. The differences among disease types depend on the timing of gut perforation during pregnancy. Meconium is clearly a strong pro-inflammatory mediator as evidenced by in vitro stimulation and clinical disease (*12-14*). In animal models, procoagulant activity and tumor necrosis factor-α (TNF-α) production by macrophages are significantly increased in response to meconium stimulation (*15*).

Several studies have evaluated the predicting factors for patients' outcome, such as the accuracy or sonographic features of prenatal diagnosis, type of MP at operation, and the patient's general condition (*2,10,16-18*). Generally, preoperative systemic inflammatory status and poor preoperative condition increase postoperative morbidity and mortality in adult patients (*19*). However, there has been no clarification on the relationship between US findings and systemic inflammatory status in the fetus or neonate with MP. In this study, we reviewed the therapy and outcome

Released online in J-STAGE as advance publication April 7, 2015.

**Address correspondence to:*
Dr. Keiichi Uchida, Department of Gastrointestinal and Pediatric Surgery, Mie University Graduate School of Medicine 2-174 Edobashi, Tsu, Mie 514-8507, Japan.
E-mail: ucchie@clin.medic.mie-u.ac.jp

for MP at our institute, and analyzed the prenatal US examination, postnatal patient's condition, and surgical outcome.

2. Patients and Methods

We retrospectively reviewed a total 15 patients with MP who received surgical intervention at the neonatal pediatric surgical unit of the Perinatal and Maternal Care Center in Mie University Hospital, Mie, Japan. Diagnosis of MP was confirmed by operative findings. MP was divided into 3 types as follows: generalized (G type), cystic (C type), and fibroadhesive type (F type) (*1*). We reviewed clinical course of our patients with MP, and analyzed predictors of outcome. Eleven of 15 patients received prenatal US examination by gynecologists. Typical findings such as dilated bowel, fetal ascites, pseudocyst and polyhydramnios just before delivery were evaluated in this study. Polyhydramnios is defined as an amniotic fluid index > 24 cm.

After birth, patients were transferred to the neonatal intensive care unit in the PMCC and evaluated by neonatologists and pediatric surgeons. Some patients received intensive care including cardio-pulmonary support. Our surgical strategy for MP is a two- or three-stage approach with abdominal drainage or temporary enterostomy and elective reconstruction of intestinal continuity (stoma closure) according to the cases. The two-stage approach consists of enterostomy and elective stoma closure. The three-stage approach consists of the abdominal drainage procedure, enterostomy and elective stoma closure. Abdominal drainage was performed using a Penrose drain or catheterization under regional or general anesthesia. Recently, the first choice for cystic type MP was abdominal decompression by catheterization closed drainage.

All patients were investigated after obtaining the parent's or guardian's informed consent to participate in this study. This study was approved by the Mie University Graduate School of Medicine Ethics Review Board.

All statistical analyses were performed using StatView software (version 5; Abacus Concepts, Berkeley, CA, USA). The results were expressed as median or means ± standard deviation (S.D.). The Mann-Whitney *U* test and Chi-square test were used for comparisons among unpaired groups. *P* values of less than 0.05 were considered statistically significant.

3. Results

The patients' characteristics are shown in Table 1. The 15 patients consisted of 9 males and 6 female babies. Four of 15 patients without prenatal diagnosis were referred to our institute if abdominal distension, bilious vomiting, or pneumoperitoneum was noted after birth. In 11 of 15 patients, prenatal diagnosis using ultrasonography (US) was made from 26 to 35 weeks of gestational age. Polyhydramnios was detected in 11 patients, bowel dilatation in 8 patients, ascites in 5 patients, and pseudocyst in 2 patients. MP consisted of 3 G types, 4 C types, and 8 F types. Causes of MP consisted of intestinal atresia in 7 patients, volvulus in 7 patients, and colon atresia in 1 patient. No associated anomaly was found in this series. None of the patients had cystic fibrosis because of the hereditary characteristics of Japanese. The median follow up period was 76 months (range, from 4 to 264 months).

Table 1. Patients' Characteristics

Patients	15
Gender (M:F)	9:6
Gestational age* (weeks and days)	36 (31w3d-39w5d)
Body weight at birth* (g)	2992 (1770-3600)
Delivery type (NVD: CS)	9:6
Apgar score at 1 min*	6 (2-10)
Admission day after birth*	1 (1-3)
Operation day after birth*	1 (1-3)
Prenatal diagnosis (yes: no)	11:4
Polyhydramnios	11 (26-35w)
Bowel dilatation	8 (26-35w)
Ascites	5 (31-33w)
Pseudocyst	2 (35w)
Type of MP; G/C/F	3/4/8
Cause of MP; V/IA/CA	7/7/1
Follow up period* (months)	76 (4-264)

*Median; NVD, normal vaginal delivery; CS, caesarian section; MP, meconium peritonitis; G, generalized; C, cystic; F, fibroadhesive; V, volvulus; IA, intestinal atresia; CA, colon atresia.

At the first surgical procedure, enterostomy was performed in 9 patients, drainage by laparotomy or catheter in 3 patients, and enterostomy after decompression in 3 patients according to postnatal US or intraoperative findings. After the 1st procedure, 5 morbidities (33.3%) occurred including wound infection in 1 patient, intestinal fistula in 1 patient, abdominal abscess in 1 patient, cerebral hemorrhage due to disseminated intravascular coagulation (DIC) in 1 patient, and sepsis in 1 patient. After the 2nd procedure, 13 patients had intact survival, 1 patient had cerebral palsy, and 1 patient died due to multiple organ failure (MOF).

In order to clarify prognostic factors, we examined the relationship between prognosis and several factors. We divided 15 patients into 2 groups according to the occurrence of morbidity after the 1st procedure; Group A without morbidity or Group B with morbidity (Table 2). There were no differences in types and causes of MP between Groups A and B. Surgical procedures had no relationship with patients' morbidities after the 1st procedure. In the preoperative condition, there were no differences between the groups for gender, gestational age, body weight at birth, delivery type, Apgar score, and prenatal diagnosis. However, there was a significant difference between the groups for preoperative presence of circulation deficiency and serum CRP values (Group

Table 2. Patients characteristics with or without morbidities

Items	Group A (n = 10) (without morbidity)	Group B (n = 5) (with morbidity)	P value
Gender (M:F)	5:5	4:01	*n.s.*
Gestational age (weeks)	36.3 ± 1.6	35.7 ± 2.8	*n.s*
Body weight at birth (g)	3025 ± 292	2746 ± 593	*n.s*
Delivery type (NVD: CS)	6:4	3:2	*n.s*
Apgar score at 1 min	6.4 ± 2.2	6.3 ± 3.8	*n.s*
Admission day after birth	1.1 ± 0.3	1.6 ± 0.9	*n.s*
Operation day after birth	1.3 ± 0.7	1.6 ± 0.9	*n.s*
Prenatal diagnosis (yes: no)	8:2	3:2	*n.s*
Preoperative condition			
Circulation failure (yes: no)	0:10	3[†]:2	0.006
Base excess of BGA	-6.7 ± 2.9	-5.4 ± 1.8	*n.s*
CRP values (mg/dL)	0.95 ± 1.0	6.7 ± 5.9	0.027
Ascites culture (positive: negative)	0:10	1[‡]:4	*n.s*
Type of MP; G/C/F	2/3/5	1/1/3	*n.s*
Cause of MP;V/IA/CA	5/5/0	2/2/1	*n.s*
Surgical procedure (E/EAD/D)	6/2/2	3/1/1	*n.s*
Follow up period* (months)	92 (13-233)	76 (12-264)	*n.s*

* Median. The Mann-Whitney U test and Chi square test were used for analysis. † hypotension 2 and persistent pulmonary hypertension 1, ‡ *E. coli* and *K. oxytoca*. NVD, normal vaginal delivery; CS, caesarean section; MP, meconium peritonitis; G, generalized; C, cystic; F, fibroadhesive; V, volvulus; IA, intestinal atresia; CA, colon atresia; BGA, blood gas analysis; CRP, C-reactive protein; E, enterostomy; EAD, enterostomy after decompression; D, drainage.

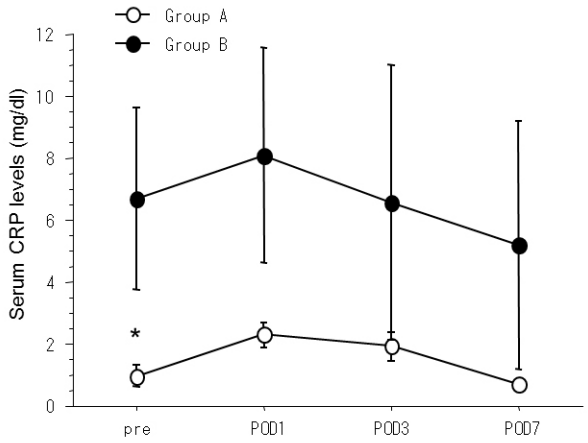


Figure 1. Serial changes in perioperative serum CRP values in Groups A and B after the 1st surgical procedure at our institute. Preoperative serum CRP values were significantly different between groups A and B (p = 0.0272).

A, p = 0.0062 and Group B, p = 0.0272, respectively). Figure 1 shows serial changes in perioperative serum CRP values. General inflammation in Group A patients was more severe than Group B patients during the perioperative period. Preoperative serum CRP values were significantly different between Groups A and B (p = 0.0272).

We present here an important case with Giant cystic type MP caused by intestinal volvulus had persistent pulmonary hypertension and systemic inflammatory syndrome preoperatively in Group B (Figure 2). After elective caesarean section (CS) because of non-reassuring fetal status with circulation deficiency, we immediately performed abdominal decompression by closed drainage catheterization (240 mL bilious discharge). After the patient's general condition was stabilized by intensive care with nitric oxide inhalation and Prostaglandin E1 administration, tube enterostomy was created under general anesthesia several hours later. After 2 weeks later, she received stoma closure operation with easy adhesiolysis and postoperative course was uneventful without wound infection and she is presently in good health.

4. Discussion

Possible causes and pathogenesis of MP include ischemia in the mesentery, volvulus, intestinal atresia, meconium plugs, internal hernia, Hirschsprung's disease, colon atresia, torsion of a fallopian tube cyst, cystic fibrosis and others (*1-7*). In this study, the most common causes of MP were intestinal atresia (47%) and volvulus (47%). This finding differs from other reports from Asian countries. Nam *et al*. (*9*) reported 31 MP patients who had intestinal atresia in 14 patients (45%), volvulus in 2 patients (6%), and uncertain ileal perforation in 10 patients (32%). Kamata *et al*. (*20*) reported 20 MP patients who had intestinal atresia in 18 patients (90%) and meconium ileus in 2 patients (10%). In our study, we also didn't find any cases of cystic fibrosis because of their rarity in Asian countries. MP can have a wide range of disease causes and clinical presentations. It is important to understand that patients in each clinical study for MP may have a different background compared with other studies elsewhere.

In our study, prenatal diagnosis was made in 73% of patients. The US findings with suspected MP were polyhydramnios (100%), bowel dilatation (53%), ascites (33%), and pseudocyst (13%). Several studies have assessed the accuracy of prenatal US examination

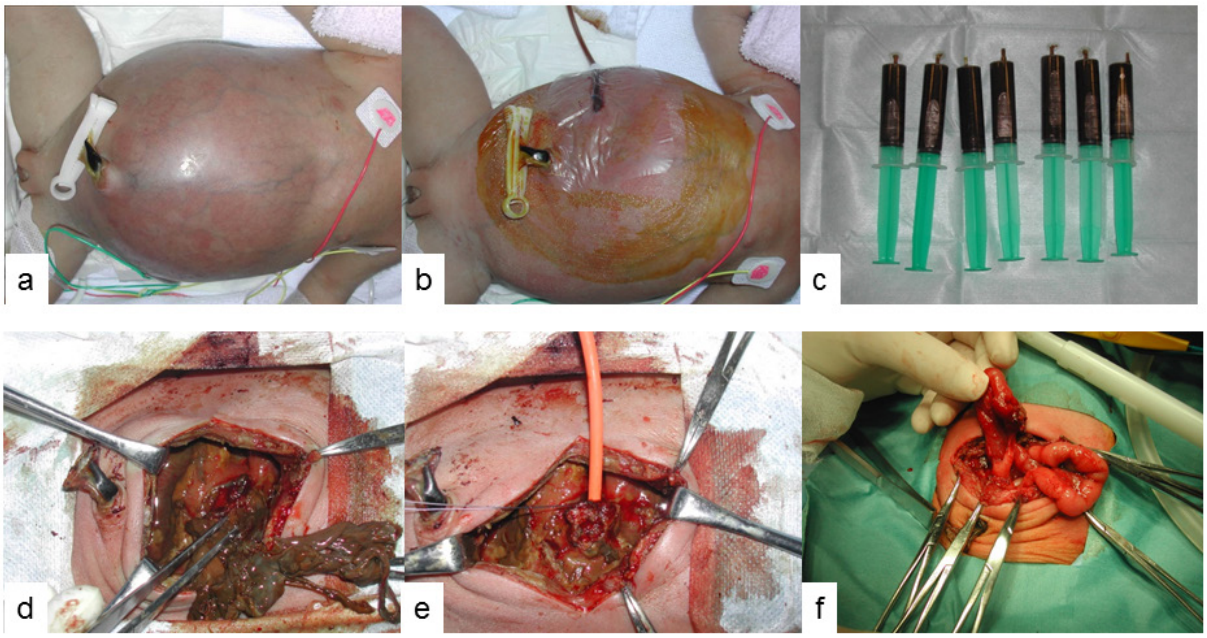


Figure 2. We present here an important case with Giant cystic type MP caused by intestinal volvulus had persistent pulmonary hypertension and systemic inflammatory syndrome preoperatively. After elective caesarean section (CS) because of non-reassuring fetal status with circulation deficiency, we immediately performed abdominal decompression by closed drainage catheterization **(a, b, c)**. After the patient's general condition was stabilized by intensive care with nitric oxide inhalation and Prostaglandin E1 administration, tube enterostomy was performed under general anesthesia several hours later **(d, e)**. After 2 weeks later, she received stoma closure operation with easy adhesiolysis **(f)** and postoperative course was uneventful without wound infection.

for diagnosing meconium peritonitis and predicting patients' outcomes (*2,16,18*). Previous reports have also demonstrated the relationship between the type of MP and patients' outcome (*9,11,20,21*). The present study did not show a significant relationship between prenatal diagnosis or types of MP and patients' outcome. A few case reports have recommended early surgical management including abdominal decompression to babies with suspected C type MP (*1,12,22*). We also recommend the surgical strategy combined with cyst drainage decompression followed by enterostomy just after birth and elective surgery at a later date for C type MP. The postnatal surgical procedure is dependent on the patients' clinical presentation and general condition.

Our surgical strategy is a two-stage approach with temporary enterostomy or drainage and delayed reconstruction of intestinal continuity. Nam *et al*. (*9*) reported that primary resection and anastomosis of the involved intestinal segment was carried out in 70% of MP patients they experienced, with an excellent morbidity rate of 2.4%. The present study demonstrated that there is no relation between surgical procedure and outcome. However, the first surgical approach after birth should be selected for complete drainage and prevention of bacterial infection.

Meconium is a complex mixture of bile salts, cell debris, and proteins. Spillage of these constituents has been shown to activate immune cells including macrophages (*23,24*). Macrophages infiltrate into the peritoneum and participate in a range of cellular functions, including phagocytosis, release of chemical mediators, and antibody-dependent cell-mediated cytotoxicity (*25*). Experimental animal studies have demonstrated that TNF-α production by macrophages is significantly increased in response to meconium stimulation (*15*). Exaggerated production of chemical mediators including TNF-α enhances fibrin deposition and severe intra-abdominal adhesion, resulting in short bowel syndrome due to massive bowel resection or poly-surgery. Moreover, if sealing of the perforation does not take place, huge abdominal cyst formation and progressive pro-inflammatory cytokine reaction with ascites collection may cause fetal cardiac insufficiency, non-reassuring fetal status, preterm labor, and a poor general condition of the infant after birth.

In this study, we demonstrated that postnatal circulation deficiency and serum CRP values are predicting factors for morbidities and mortalities of MP patients. Shyu *et al*. (*18*) also demonstrated that persistent ascites and postnatal persistent pulmonary hypertension of newborns significantly correlate with neonatal mortality. The present study also demonstrated that fetuses with a severe systemic inflammatory status were likely to receive Caesarean section due to non-reassuring fetal status or require intensive care due to cardio-pulmonary insufficiency after birth. Moreover, they had a high morbidity rate after postnatal clinical course compared with fetuses with no inflammatory status. In order to improve the critical condition of patients, surgical intervention in utero such as fetal paracentesis

may be beneficial by reducing intraabdominal pressure and removing inflammatory debris and cytokines (*26,27*).

In conclusion, prenatal diagnosis is essential for the first step of perinatal therapy for MP. Timing of delivery and fetal intervention according to fetal conditions should be discussed with gynecologists, neonatologists, and neonatal pediatric surgeons in perinatal and maternal care centers. The surgical strategy should be selected according to the information of prenatal diagnosis. Proper surgical procedures for reducing systemic and abdominal inflammation after birth may improve the outcome of severe MP cases.

References

1. Lorimer WS Jr, Ellis DG. Meconium peritonitis. Surgery. 1966; 60:470-475.
2. Chan KL, Tang MH, Tse HY, Tang RY, Tam PK. Meconium peritonitis: Prenatal diagnosis, postnatal management and outcome. Prenat Diagn. 2005; 25:676-682.
3. Hamada T, Hirose R, Kosaka T, Taniguchi K, Noguchi M, Kihara T, Egashira M, Tagawa M, Miura K, Masuzaki H, Tajima Y, Hayashi T, Kanematsu T. Giant cystic meconium peritonitis associated with a cloacal anomaly: Case report. J Pediatr Surg. 2008; 43:E21-23.
4. Reynolds E, Douglass B, Bleacher J. Meconium peritonitis. J Perinatol. 2000; 20:193-195.
5. Bendel WJ Jr, Michel ML Jr. Meconium peritonitis; review of the literature and report of a case with survival after surgery. Surgery. 1953; 34:321-333.
6. Heydenrych JJ, Marcus PB. Meconium granulomas of the tunica vaginalis. J Urol. 1976; 115:596-598.
7. Agrawala G, Predanic M, Perni SC, Chasen ST. Isolated fetal ascites caused by bowel perforation due to colonic atresia. J Matern Fetal Neonatal Med. 2005; 17:291-294.
8. Payne RM, Nielsen AM. Meconium peritonitis. Am Surg. 1962; 28:224-231.
9. Nam SH, Kim SC, Kim DY, Kim AR, Kim KS, Pi SY, Won HS, Kim IK. Experience with meconium peritonitis. J Pediatr Surg. 2007; 42:1822-1825.
10. Tseng JJ, Chou MM, Ho ES. Meconium peritonitis in utero: Prenatal sonographic findings and clinical implications. J Chin Med Assoc. 2003; 66:355-359.
11. Kuroda T, Kitano Y, Honna T, Sago H, Hayashi S, Saeki M. Prenatal diagnosis and management of abdominal diseases in pediatric surgery. J Pediatr Surg. 2004; 39:1819-1822.
12. Tanaka K, Hashizume K, Kawarasaki H, Iwanaka T, Tsuchida Y. Elective surgery for cystic meconium peritonitis: Report of two cases. J Pediatr Surg. 1993; 28:960-961.
13. Agerty HA, Ziserman AJ, Schollenberger CL. A case of perforation of the ileum in a newborn infant with operation and recovery. The Journal of Pediatrics. J Pediatr. 1943; 22:233-238.
14. Andrassy RJ, Nirgiotis JG. Meconium disease of infancy: Meconium ileus, meconium plug syndrome, and meconium peritonitis. (Holder RM, Ashcraft KW, eds): Pediatric Surgery. Philadelphia, PA, Saunders, USA, 1993, pp. 338-339.
15. Lally KP, Mehall JR, Xue H, Thompson J. Meconium stimulate a pro-inflammatory response in peritoneal macrophages: Implications for meconium peritonitis. J Pediatr Surg. 1999; 34:214-217.
16. Zangheri G, Andreani M, Ciriello E, Urban G, Incerti M, Vergani P. Fetal intra-abdominal calcifications from meconium peritonitis: Sonographic predictors of postnatal surgery. Prenat Diagn. 2007; 27:960-963.
17. Eckoldt F, Heling KS, Woderich R, Kraft S, Bollmann R, Mau H. Meconium peritonitis and pseudo-cyst formation: Prenatal diagnosis and post-natal course. Prenat Diagn. 2003; 23:904-908.
18. Shyu MK, Shih JC, Lee CN, Hwa HL, Chow SN, Hsieh FJ. Correlation of prenatal ultrasound and postnatal outcome in meconium peritonitis. Fetal Diagn Ther. 2003; 18:255-261.
19. Uchida K, Miki C, Urata H, Kusunoki M. Serum concentration of hepatocyte growth factor predicts perioperative surgical stress in children. Eur J Surg. 2001; 167:97-101.
20. Kamata S, Nose K, Ishikawa S, Usui N, Sawai T, Kitayama Y, Okuyama H, Imura K, Okada A. Meconium peritonitis in utero. Pediatr Surg Int. 2000; 16:377-379.
21. Cerise EJ, Whitehead W. Meconium peritonitis. Am Surg. 1969; 35:389-392.
22. Pelizzo G, Codrich D, Zennaro F, Dell'oste C, Maso G, D'Ottavio G, Schleef J. Prenatal detection of the cystic form of meconium peritonitis: No issues for delayed postnatal surgery. Pediatr Surg Int. 2008; 24:1061-1065.
23. Shyu MK, Chen CD, Hsieh FJ, Yau KI, Lin GJ, Lee ML. Intrauterine intervention in a case of recurrent meconium peritonitis. Prenat Diagn. 1994; 14:993-995.
24. Rubin BK, Tomkiewicz RP, Patrinos ME, Easa D. The surface and transport properties of meconium and reconstituted meconium solutions. Pediar Res. 1996; 40:834-838.
25. Ramin KD, Leveno KJ, Kelly MA, Carmody TJ. Amniotic fluid meconium: A fetal environmental hazard. Obstet Gynecol. 1996; 87:181-184.
26. Topley N, Mackenzie RK, Williams JD. Macrophages and mesothelial cells in bacterial peritonitis. Immunobiology. 1996; 195:563-573.
27. Okawa T, Soeda S, Watanabe T, Sato K, Sato A. Repeated paracentesis in a fetus with meconium peritonitis with massive ascites: A case report. Fetal Diagn Ther. 2008; 24:99-102.

(Received March 20, 2015; Revised March 26, 2015; Accepted April 3, 2015;)

Original Article

DOI: 10.5582/irdr.2015.01010

Functional recovery after acute intravenous administration of human umbilical cord mesenchymal stem cells in rats with cerebral ischemia-reperfusion injury

Dongmei Li[1], Min Zhang[1,*], Qiuhua Zhang[2], Yue Wang[1], Xuxia Song[1], Qiuling Zhang[3]

[1] *Department of Neurology, Qingdao Municipal Hospital, Qingdao, Shandong, China;*
[2] *Department of Rehabilitation, Shuangshan Hospital of the Zibo Mining Group, Zibo, Shangdong, China;*
[3] *Department of Physiology, Taishan Medical College, Taian, Shandong, China.*

Summary **Cell therapy is a potential approach for treatment of strokes. Mesenchymal stem cells (MSCs) are a potential cell source for clinical use because they are safe and easy to obtain. A peptide solution can promote neural regeneration. Previously, such a solution was stereotactically injected into the brain of rats with cerebral infarction, resulting in improvement in the animal's neurological function and reduction in the infarction volume, but the injury was relatively severe. The current study established a rat model of cerebral ischemia-reperfusion (I/R) injury. MSCs isolated from Wharton's jelly of human umbilical cords (HUMSCs) were injected intravenously immediately after cerebral I/R injury(3×10^6 cells per rat). Twenty-four h and 14 d after surgery, animal behavior was evaluated using the Rogers test and infarct lesion volume was evaluated by 2,3,5-triphenyltetrazolium chloride staining. Fourteen d after surgery, brain tissues were collected at 14 d to study migration/implantation of HUMSCs, cellular proliferation, neural regeneration and astrocyte activation. Compared to cerebral I/R injury alone, HUMSC treatment improved function at 14 d after surgery, with no reduction in infarct volume or migration or implantation of cells into the damaged brain areas. Nevertheless, 14 d after surgery, HUMSC administration increased cellular proliferation and the level of neurofilament 200 level and decreased the level of glial fibrillary acidic protein. After cerebral I/R injury, acute intravenous administration of HUMSCs could promote recovery by activating endogenous neural regeneration and inhibiting astrocyte activation, without migration and implantation directly into lesions.**

Keywords: Cerebral ischemia-reperfusion, human umbilical cord mesenchymal stem cell, cellular proliferation, neural regeneration, astrocyte activation, neurofilament protein 200, glial fibrillary acidic protein

1. Introduction

Stroke is a major cause of death and disability in adults worldwide. Despite decades of intense research, current treatments for acute ischemia stroke are far from optimal (*1*). Ischemia stroke triggers a cascade of pathophysiological events, including excitotoxicity, oxidative stress, apoptosis and inflammation (*2*). In addition, neural regeneration, synaptogenesis, astrocyte activation and angiogenesis are stimulated within the brain post-stroke (*3,4*); however, neural regeneration is markedly reduced in the aged rodent brain (*5*). In recent years, experimental studies and clinical trials have indicated that cell-based therapiesoffer promise as a stroke treatment.

One of the main challenges is to identify the best source of stem cells for use after stroke. Transplantation of stem cells from fetal tissues and progenitor cells from bone marrow and human umbilical cord blood has promoted neuronal survival, tissue repair, and, most importantly, recovery after experimental stroke (*6*).

**Address correspondence to:*
Dr. Min Zhang, Department of Neurology, Qingdao Municipal Hospital, Qingdao, No.1, Jiaozhou Road, 266011 Qingdao, Shandong, China.
E-mail: dongmeili1999@126.com

However, limited sources of stem and progenitor cells as well as ethical and safety concerns associated with the use of embryonic stem cells have spurred a search for alternative stem cell sources for stroke treatment. Mesenchymal stem cells (MSCs) have emerged as a leading candidate source for stroke management; MSCs are easily obtained from a range of tissues (*7*), can be rapidly expanded *in vitro* while maintaining their potential to differentiate into multiple tissues, and are immune-privileged, with reduced risk of rejection (*8*). MSCs isolated from Wharton's jelly of human umbilical cords (HUMSCs) can escape immune surveillance possibly because of the absence of the major histocompatibility complex II antigen and co-stimulatory surface antigens CD40, CD80, and CD86 (*9,10*).

Originally, thinking was that stem cells had to be placed within the damaged sites of the brain after ischemia to promote recovery. In many studies, different routes have been tested, including intrastriatal (*11*), intracerebral (*12*), intraventricular (*13*), and intrathecal by lumbar puncture (*14*). However, these techniques raise the possibility of additional trauma from transplantation surgery and are often impractical for patients with clinically serious conditions. Recent studies have demonstrated that cell-based therapy is not intended to replace damaged cells but rather to remodel the central nervous system by promoting neuroplasticity, angiogenesis, and immunomodulation (*15,16*).

The optimal timing for the administration of stem cells to treat cerebral ischemia is another main challenge. Because of the time needed to prepare autologous stem cells after an unexpected stroke, such cells can only be administered several weeks later, and previous studies have focused on post-acute-phase intervention (*16,17*). Promising experimental animal data suggest that early stem cell administration can interrupt the initiation of the very beginning of the ischemic cascade (*15*). Despite several reports of the treatment of experimental cerebral ischemia with bone-marrow-derived MSCs (BM-MSCs) and adipose tissue-derived MSCs (AD-MSCs) in the acute phase (*18,19*), there are no reports of the acute intravenous administration of HUMSCs to treat cerebral ischemia.

Here, an established rat model of middle cerebral artery occlusion (MCAO) was used to investigate the potential therapeutic effects of acute intravenous administration of HUMSCs on cerebral ischemia-reperfusion (I/R) injury.

2. Materials and Methods

2.1. *Animals*

A randomized, controlled animal experiment was performed at the Experimental Animal Center and Neurobiology Laboratory of Taishan Medical College, China from September 2012 to June 2013. In total, 54 Sprague Dawley pathogen-free rats of both sexes weighing 250-300 g and 12-14 weeks old were provided by the Experimental Animal Center of Shandong Provincial University of Traditional Chinese Medicine (License No. SCXK [Lu] 2005-0015). Rats were housed at 24 ± 3°C in 40% to 70% relative humidity under a 12-h light-dark illumination cycle and had free access to food and water. Experimental protocols were in accordance with Guidance Suggestions for the Care and Use of Laboratory Animals, formulated by the Ministry of Science and Technology of the People's Republic of China.

2.2. *Human umbilical cords*

Human umbilical cords were obtained from full-term deliveries with informed consent from parents after caesarian section. The procedure for collecting tissues was approved by the ethics committee of the Second Hospital of Shandong University.

2.3. *HUMSC isolation and in vitro culture*

Isolation and culture of HUMSCs were as described. After arteries and veins were removed, the remaining tissue, Wharton's jelly, was cut into 0.5-1 cm^3 pieces and suspended in Dulbecco's modified Eagle's low-glucose media (DMEM-LG; Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco, USA), 100 mg/mL penicillin, and 100 mg/mL streptomycin. The tissue was left undisturbed for 7 days in a 37°C humidified incubator with 5% CO_2 to allow cells to migrate from the explants. Culture medium was replaced every 3 days. The cells were passaged through use of a 0.25% trypsin-EDTA solution when cells reached 80% to 90% confluence. The identification of HUMSCs by flow cytometry was as previously described. The cells used in this study were positive for CD73, CD90, and CD105 but negative for CD34, CD45, and HLA-DR, which is consistent with the characteristics of HUMSCs.

2.4. *Animal groups*

The rats were randomly assigned to 3 groups for treatment (n = 18 animals each): sham-surgery without cerebral I/R injury and infusion of phosphate-buffered saline (PBS) *via* the tail vein; cerebral I/R injury induced by MCAO and reperfusion and infusion of PBS *via* the tail vein; and MCAO and reperfusion and infusion of HUMSC *via* the tail vein.

2.5. *Preparation of a model of cerebral I/Rinjury*

As previously described, rats were anesthetized with an intraperitoneal injection of 10% (w/v) chloral hydrate (350 mg/kg; Boster, Wuhan, China). A 2-cm incision was made in the middle of the neck line, separating the

right carotid artery, as well as the internal and external carotid communicating arteries. Through a small incision to the external carotid artery, a previously selected plug thread was inserted from the right external carotid artery into the right internal carotid artery to a depth of 18.0 ± 0.5 mm (beginning with the common carotid artery bifurcation); the thread was maintained for 2 h and then removed to restore blood flow to the common carotid and internal carotid arteries. Body temperature was maintained at 37 ± 0.5°C with a heating lamp linked to a rectal thermometer during all phases of the surgery. Under anesthesia, rats in the sham surgery group only had the common carotid artery, internal carotid artery, and external carotid artery exposed.

2.6. *Cell administration*

A HUMSC suspension or PBS was injected *via* the tail vein at the onset of reperfusion. Before the injection, the cell suspension was washed with PBS 3 times to remove serum. Intravenous injections of 3×10^6 HUMSCs in 600 μL saline were administered *via* the tail vein over 4 min. Cerebral I/R injury was induced in animals with MCAO and reperfusion, but animals only received an equal volume of PBS at that time. Cerebral I/R injury was not induced in sham-operated animals, and animals received an injection of PBS *via* the tail vein. Sham-operated animals and animals with cerebral I/R injury both received an equal volume of PBS without HUMSCs over 4 min.

2.7. *BrdU incorporation*

All animals were given 50 mg/kg of daily intraperitoneal 5-bromo-2'-deoxyuridine (BrdU, Sigma-Aldrich) on days 4 to 7 after cerebral I/R injury . This administration protocol was based on previous reports of proliferation peaking 4 to 10 days after injury.

2.8. *Functional evaluation*

All animals were functionally evaluated 24 h and 14 d after surgery usingthe Rogers scale: 0, no deficit; 1, failure to extend left forepaw; 2, decreased grip of the left forelimb when the tail is pulled; 3, spontaneous movement in all directions, contralateral circling if pulled; 4, circling or walking to the left; 5, movement only when stimulated; 6, unresponsive to stimulation; and 7, dead.

2.9. *Measurement of cerebral infarct volume*

Twenty-four h and 14 d after surgery, cerebral infarct volume was measured in 6 animals from each group. Rats were decapitated, their brains were rapidly removed and cut into 6 2-mm-thick coronal sections, and sections were stained with 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution at 37˚C for 30 min. Stained slides were washed in PBS for 5 min and immersed overnight in 4% paraformaldehyde. Unstained areas were defined as infarcted tissue, and normal tissue was stained red. The total infarct volume was the sum of the infarcted areas in the 6 sections and was a percentage of cerebral ischemic volume in the hemisphere ipsilateralto the lesion.

2.10. *Histological staining*

Fourteen d after surgery, 6 animals from each group were used for histological staining. Rats were deeply anesthetized with chloral hydrate, then cardiac perfused immediately with ice-cold PBS and 4% paraformaldehyde in 0.1 M PBS (pH 7.4) after functional evaluation. Brains were excised and post-fixed overnight at 4°C and then incubated in 30% sucrose at 4°C until equilibration (5 mice per group). Coronal sections 10 μm thick were prepared using of a freezing microtome (LEICA CM1850; Germany) and were stored at -20°C.

Immunohistochemistry was performed as described. Brain sections were treated with 2 M HCL for 30 min at 37.0°C. Sections were washed in PBS, blocked in PBS containing 0.4% Triton X-100 (Sigma) and 5% goat serum (Jackson ImmunoResearch Lab) for 1 h, and then incubated with the primary antibody at 4°C: rabbit monoclonal anti-HuN (Chemicon, 1:300),overnight at 4°C to analyze the migration and implantation of HUMSCs in rat brains or rabbit anti-BrdU (1:500, Sigma) to detect cellular proliferation in brains. Sections were then exposed to biotinylated goat anti-rabbit IgG (1:200) at room temperature for 10 min, followed by exposure to biotin-streptavidin-alkaline phosphatase at room temperature for 10 min.

Immunofluorescence was performed as described to detect neural regeneration and astrocyte activation. Sections underwent double staining with mouse anti-neurofilament 200 (NF200) monoclonal antibody (1:100, Abnova; a marker of neural regeneration) followed by goat anti-mouse Alexa Fluor 488 (1: 750, Molecular Probes, Invitrogen) and rabbit anti-BrdU (1:500, Sigma) and then goat anti-rabbit Alexa Fluor 594 (1:750, Molecular Probes, Invitrogen). Sections were stained with mouse anti-glial fibrillary acid protein (GFAP) monoclonal antibody (1:400, Chemicon; a marker of astrocyte activation) followed by goat anti-mouse Alexa Fluor 488. Finally, sections were coverslipped with medium for fluorescence (Vector Labs) and viewed usingfluorescence microscopy (Olympus, Japan).

2.11. *Statistical analysis*

All data are expressed as mean ± S.D. Differences were analyzed using analysis variance (ANOVA) and the Student's *t*-test in SPSS 16.0 for Windows (SPSS Inc., Chicago, IL, USA). p value < 0.05 was considered statistically significant.

3. Results

3.1. *Intravenous administration of HUMSCs improved functional recovery after cerebral I/R injury*

Animals were clinically evaluated 24 h and 14 d after surgery using the Rogers score. Sham-operated rats did not display a functional deficit 24 h or 14 d after surgery. Twenty-four h after surgery, the Rogers score did not differ for rats treated with HUMSCs and untreated rats with cerebral I/R injury ($p = 0.307$; Figure 1A), but 14 d after surgery, rats treated with HUMSCs had better functional recovery than did untrreated rats with cerebral I/R injury ($p = 0.016$).

3.2. *Infarct size after cerebral I/R injury did not change after intravenous HUMSC administration*

TTC staining was used to evaluate Infarct lesions 24 h and 14 d after the experimental procedure. by Staining revealed that brains from sham-operated rats had no lesions. Both rats treated with HUMSCs and untreated rats with cerebral I/R injury had fewer lesions 14 d after the procedure than they did 24 h afterwards. There were no differences in the number of lesions in the 2 groups at either time ($p = 0.801$ and $p = 0.626$, respectively, Figure 1B).

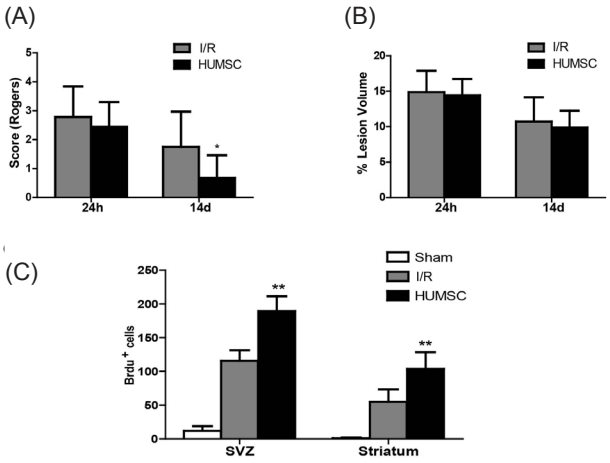


Figure 1. (A), Intravenous administration of HUMSCs improved functional recovery after cerebral I/R injury. Animals were clinically evaluated 24 h and 14 d after surgery using the Rogers score. Sham-operated rats did not display a functional deficit 24 h and 14 d after surgery. Twenty-four h after surgery, the Rogers score did not differ for rats treated with HUMSCs and untreated rats with cerebral I/R injury ($p = 0.307$),but 14d after surgery rats treated with HUMSCs had better functional recovery than did untreated rats with cerebral I/R injury($p = 0.016$). **(B), Infarct size after cerebral I/R injury did not change after intravenous HUMSC administration.** TTC staining was used to evaluate infarct lesions24 h and 14 d after the experimental procedure, This staining revealed that brains from sham-operated rats had no lesions. Both rats treated with HUMSCs and untreated rats with cerebral I/R injury had fewer lesions 14d than 24h after the prodedure, and the number of lesions in the two groups did not differ at either time($p = 0.801$ and $p = 0.626$,respectively). **(C), Intravenous administration of HUMSC increased cellular proliferation after cerebral I/R injury.** Cellular proliferation was detected with BrdU immunohistochemical staining of rat brain sections. Brain sections from sham-operated rats had only a few BrdU-positive cells. Fourteen d after surgery, brain sections from rats treated with HUMSCs had a greater number of BrdU-positive cells in both the subventricular zone (SVZ; $p < 0.001$) and striatum ($p = 0.001$) than did untreated rats with cerebral I/R injury.

3.3. *Intravenous administration of HUMSCs did not lead to migration or implantation of HUMSCs*

Migration and implantation of HUMSCs was studied using immunohistochemistry with anti-human nuclear antigen (HuN) antibody. Staining was not evident in brain sections from sham-operated rats and untreated rats with cerebral I/R injury. However, brain sections from rats treated with HUMSCs displayed neither migration nor implantation at 14 d after treatment (data not shown).

3.4. *Intravenous administration of HUMSC increased cellular proliferation after cerebral I/R injury*

Cellular proliferation was detected with BrdU immunohistochemical staining of rat brain sections. Brain sections from sham-operated rats had only a few BrdU-positive cells (Figure 1C). Brain sections from rats treated with HUMSCs had a greater number of BrdU-positive cells in both the subventricular zone (SVZ; $p < 0.001$) and striatum ($p = 0.001$) than did untreated rats with cerebral I/R injury 14 d after the procedure.

3.5. *Intravenous administration of HUMSCs increased endogenous neural regeneration after cerebral I/R injury*

Since there was no migration and implantation of HUMSCs in rats treated with HUMSCs, endogenous neural regeneration was studied in brain sections 14 d after the procedure using immunofluorescence double staining with neurofilament 200 (NF200) and BrdU (Figure 2). Cells double-stained with NF200 and BrdU were not evident in brain sections from sham-operated rats. Cell double-stained with NF200 and BrdU were evident in the SVZ but not in the striatum of brain sections from untreated rats with cerebral I/R injury. As expected, cells double-stained with NF200 and BrdU were evident in both the SVZ and striatum of brains from rats treated with HUMSCs.

3.6. *Intravenous administration of HUMSCs attenuated astrocyte activation after cerebral I/R injury*

GFAP was used as an immunofluorescence marker of astrocyte activation in the striatum of rats (Figure 2). Rats with cerebral I/R injury had a greater number of GFAP-positive cells than did sham-operated rats and rats treated with HUMSCs had fewer GFAP- positive cells than did untreated rats with cerebral I/R injury.

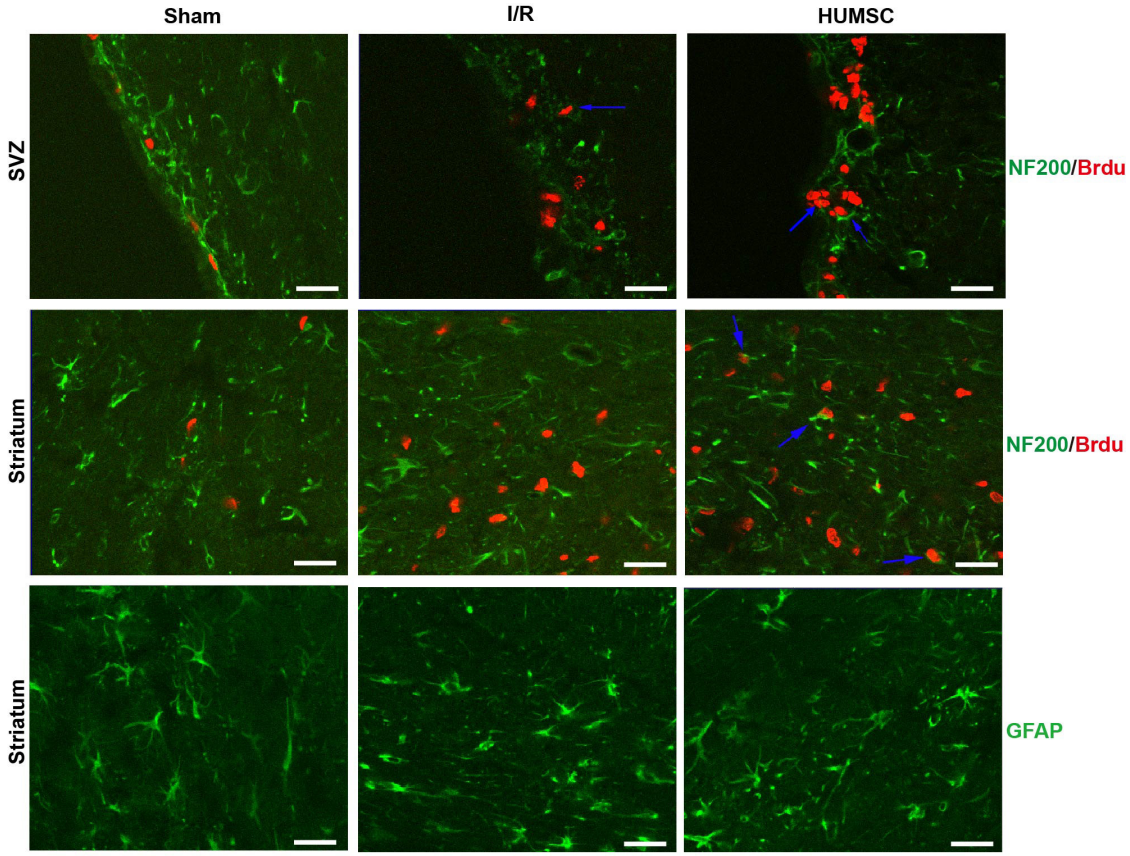


Figure 2. Intravenous administration of HUMSCs increased endogenous neural regeneration after cerebral I/R injury. Since there was no migration and implantation of HUMSCs in rats treated with HUMSCs, endogenous neural regeneration was studied using immunofluorescence double staining with neurofilament 200 (NF200) and BrdU in brain sections 14 d after surgery. Cells double-stained with NF200 and BrdU were not evident in brains sections from sham-operated rats. Cells double-stained with NF200 and BrdU were evident in the SVZ but not in the striatum of brain sections from untreated rats with cerebral I/R injury. As expected, neuronal cells double-stained with NF200 and BrdU were evident in both the SVZ and striatum of brains from rats treated with HUMSCs. **Intravenous administration of HUMSCs attenuated astrocyte activation after cerebral I/R injury.** Glial fibrillary acidic protein (GFAP) was used as an immunofluorescence marker of astrocyte activation in the striatum of rats. Untreated rats with cerebral I/R injury had a greater number of GFAP-positive cells than did sham-operated rats and rats treated with HUMSCs had fewer GFAP-positive cells than did untreated rats with cerebral I/R injury.

4. Discussion

This study investigated the effects of acute intravenous administration of HUMSCs in rats 24 h and 14 d after cerebral I/R injury. The treatment significantly improved functional recovery but did not reduce the infarct volume or cause migration or implantation of HUMSCs in damaged brain areas. Animals receiving HUMSCs had increased cell proliferation, a higher level of NF200 (a marker of neural regeneration), as well as a reduced level of GFAP (a marker of astrocyte activation) in the brain 14 d after cerebral I/R injury. A point worth noting is that acute intravenous administration of HUMSCs might have clinical implications for translation to human trials.

In a recent study of permanent MCAO in rats, the authors reported efficient functional recovery with intravenous administration of AD-MSCs and BM-MSCs (*18*). In the current study, the functional scores 14 d after cerebral I/R injury improved significantly with intravenous administration of HUMSCs. Twenty-four h and 14 d after cerebral I/R injury, there were no differences in TTC staining in rats treated with HUMSCs and untreated rats with cerebral I/R injury. HUMSCs administered intravenously did not migrate to or implant in the brain according to immunohistochemistry. Therefore, stem cells might not need to migrate and graft onto the lesion site for a positive functional result because of the paracrine effects of MSCs (*20*). This favorable outcome could be a consequence of the secretion of several growth factors that may act to enhance endogenous repair mechanisms normally activated in the brain after stroke. Such repair mechanisms include endogenous neural regeneration, synaptogenesis, astrocyte inactivation and immunomodulation (*21,22*).

Compared to cerebral I/R injury alone, intravenous administration of HUMSCs increased cellular proliferation in the SVZ and striatum of brains 14 d after cerebral I/R injury. The current results agree with those of previous studies reporting increases in cellular proliferation after AD-MSC and BM-MSC administration (*18,23*). In line with earlier studies, a later study found that, compared to infarction alone, administration of MSCs promoted cellular proliferation in SVZ and cell differentiation into neuroblasts in peri-

infarct areas (*24*).

After a stroke, neuroblasts generated in the SVZ migrate to the ischemic boundary zone (IBZ) and actively interact with the microenvironment to reach the ischemic striatum individually or in chains. After migration, SVZ-derived neuroblasts differentiate into mature neurons in the IBZ (*3*). Levels of NF expression increase 7 d after focal cerebral ischemia in the IBZ (*25*). A small number of studies have documented increased neural regeneration in the SVZ or IBZ with transplantation of non-neuronal cells in rodents after stroke (*26*). The present study, noted significantly increased levels of NF200 in the SVZ after cerebral I/R injury and in both the SVZ and striatum following HUMSC administration 14 d after surgery, so neural regeneration after ischemia was enhanced by intravenous HUMSC administration.

Brain tissue repair is a dynamic ongoing process involving reactive astrocytes, microglia, and oligodendrocytes after stroke injury. In the early stage of cerebral ischemic injury, activated astrocytes have a protective effect on brain tissue by inactivating glutamate-mediated excitotoxicity and oxidative stress. However, astrocytes are then involved in hindering axonal regeneration because of glial scar formation. GFAP is commonly used as a marker of astrogliosis and astrocyte activation in several conditions involving cerebral ischemia (*27*). In the current study, significantly increased levels of GFAP were noted in the striatum after cerebral I/R injury, and these levels then decreased with intravenous administration of HUMSCs. This finding agrees with the results of previous studies using other types of MSCs (*16,18*).

Inthe current study, acute intravenous administration of HUMSCs following cerebral I/R injury promoted recovery with neural regeneration and astrocyte inactivation and no migration or implantation of cells into lesions. Future studies are needed to determine the cell fate after intravenous administration of HUMSCs, the mechanism of action for HUMSCs, and the optimal dose to avoid undesired adverse reactions.

Acknowledgements

The manuscript is original, has not been submitted to or is not under consideration by another publication, has not been previously published in any language or any form, including electronic, and contains no disclosure of confidential information or authorship/patent application/funding source disputations

References

1. Kunz A, Dirnagl U, Mergenthaler P. Acute pathophysiological processes after ischaemic and traumatic brain injury. Best Pract Res Clin Anaesthesiol. 2010; 24:495-509.
2. Jauch EC, Saver JL, Adams HP Jr, *et al*. Guidelines for the early management of patients with acute ischemic stroke: A guideline for healthcare professionals from the American Heart Association/American Stroke Association. Stroke. 2013; 44:870-947.
3. Xiong Y, Mahmood A, Chopp M. Angiogenesis, neurogenesis and brain recovery of function following injury. Curr Opin Investig Drugs. 2010; 11:298-308.
4. Namura S, Ooboshi H, Liu J, Yenari MA. Neuroprotection after cerebral ischemia. Ann N Y Acad Sci. 2013; 1278:25-32.
5. Jin K, Minami M, Xie L, Sun Y, Mao XO, Wang Y, Simon RP, Greenberg DA. Ischemia-induced neurogenesis is preserved but reduced in the aged rodent brain. Aging Cell. 2004; 3:373-377.
6. Zhang J, Chopp M. Cell-based therapy for ischemic stroke. Expert Opin Biol Ther. 2013; 13:1229-1240.
7. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physio. 2007; 213:341-347.
8. Lalu MM, McIntyre L, Pugliese C, Fergusson D, Winston BW, Marshall JC, Granton J, Stewart DJ; Canadian Critical Care Trials Group. Safety of cell therapy with mesenchymal stromal cells (Safe Cell): A systematic review and meta-analysis of clinical trials. PLoS One. 2012; 7:e47559.
9. Weiss ML, Anderson C, Medicetty S, Seshareddy KB, Weiss RJ, VanderWerff I, Troyer D, McIntosh KR. Immune properties of human umbilical cord Wharton's jelly-derived cells. Stem Cells. 2008; 26:2865-2874.
10. Bongso A, Fong CY. The therapeutic potential, challenges and future clinical directions of stem cells from the Wharton's jelly of the human umbilical cord. Stem Cell Rev. 2013; 9:226-240.
11. Denham M, Parish CL, Leaw B, Wright J, Reid CA, Petrou S, Dottori M, Thompson LH. Neurons derived from human embryonic stem cells extend long-distance axonal projections through growth along host white matter tracts after intra-cerebral transplantation. Front Cell Neurosci. 2012; 6:11.
12. Fan HJ, Yu WD, Wang ZL, Wang Q, He ZY. Ultra-early treatment of bone marrow-derived mesenchymal stem cells for focal cerebral ischemia/reperfusion injury. Neural Regeneration Res. 2010; 5:1296-1302.
13. Jin K, Sun Y, Xie L, Mao XO, Childs J, Peel A, Logvinova A, Banwait S, Greenberg DA. Comparison of ischemia-directed migration of neural precursor cells after intrastriatal, intraventricular, or intravenous transplantation in the rat. Neurobiol Dis. 2005; 18:366-374.
14. Lim JY, Jeong CH, Jun JA, Kim SM, Ryu CH, Hou Y, Oh W, Chang JW, Jeun SS. Therapeutic effects of human umbilical cord blood-derived mesenchymal stem cells after intrathecal administration by lumbar puncture in a rat model of cerebral ischemia. Stem Cell Res Ther. 2011; 2:38.
15. Gutiérrez-Fernández M, Fuentes B, Rodríguez-Frutos B, Ramos-Cejudo J, Vallejo-Cremades MT, Díez-Tejedor E. Trophic factors and cell therapy to stimulate brain repair after ischaemic stroke. J Cell Mol Med. 2012; 16:2280-2290.
16. McGuckin CP, Jurga M, Miller AM, *et al*. Ischemic brain injury: A consortium analysis of key factors involved in mesenchymal stem cell-mediated inflammatory reduction. Arch Biochem Biophys. 2013; 534:88-97.
17. van Velthoven CT, Kavelaars A, van Bel F, Heijnen CJ. Regeneration of the ischemic brain by engineered stem cells: Fuelling endogenous repair processes. Brain Res Rev. 2009; 61:1-13.

18. Gutiérrez-Fernández M, Rodríguez-Frutos B, Ramos-Cejudo J, Teresa Vallejo-Cremades M, Fuentes B, Cerdán S, Díez-Tejedor E. Effects of intravenous administration of allogenic bone marrow- and adipose tissue-derived mesenchymal stem cells on functional recovery and brain repair markers in experimental ischemic stroke. Stem Cell Res Ther. 2013; 4:11.
19. Gutiérrez-Fernández M, Rodríguez-Frutos B, Alvarez-Grech J, Vallejo-Cremades MT, Expósito-Alcaide M, Merino J, Roda JM, Díez-Tejedor E. Functional recovery after hematic administration of allogenic mesenchymal stem cells in acute ischemic stroke in rats. Neuroscience. 2011; 175:394-405.
20. Bunnell BA, Betancourt AM, Sullivan DE. New concepts on the immune modulation mediated by mesenchymal stem cells. Stem Cell Res Ther. 2010; 1:34.
21. Li Y, Chopp M. Marrow stromal cell transplantation in stroke and traumatic brain injury. Neurosci Lett. 2009; 456:120-123.
22. Liew A, O'Brien T. Therapeutic potential for mesenchymal stem cell transplantation in critical limb ischemia. Stem Cell Res Ther. 2012; 3:28.
23. Leu S, Lin YC, Yuen CM, Yen CH, Kao YH, Sun CK, Yip HK. Adipose-derived mesenchymal stem cells markedly attenuate brain infarct size and improve neurological function in rats. J Transl Med. 2010; 8:63.
24. Yoo SW, Kim SS, Lee SY, Lee HS, Kim HS, Lee YD, Suh-Kim H. Mesenchymal stem cells promote proliferation of endogenous neural stem cells and survival of newborn cells in a rat stroke model. Exp Mol Med. 2008; 40:387-397.
25. Schroeder E, Vogelgesang S, Popa-Wagner A, Kessler C. Neurofilament expression in the rat brain after cerebral infarction: Effect of age. Neurobiol Aging. 2003; 24:135-145.
26. Jin K, Xie L, Mao X, Greenberg MB, Moore A, Peng B, Greenberg RB, Greenberg DA. Effect of human neural precursor cell transplantation on endogenous neurogenesis after focal cerebral ischemia in the rat. Brain Res. 2011; 1374:56-62.
27. Ricci G, Volpi L, Pasquali L, Petrozzi L, Siciliano G. Astrocyte-neuron interactions in neurological disorders. J Biol Phys. 2009; 35:317-336.

(Received March 15, 2015; Revised April 16, 2015; Accepted April 22, 2015)

Case Report

DOI: 10.5582/irdr.2015.01006

Abdominal pregnancy: Methods of hemorrhage control

Shipra Kunwar[1,*], Tamkin Khan[2], Kumkumrani Srivastava[1]

[1] *Era's Lucknow Medical College,Obstetrics and Gynaecology,Lucknow, India;*
[2] *Department of OBG, Obstetrics and Gynaecology, Jawaharlal Nehru Medical College, A.M.U, Aligarh, India.*

Summary **Abdominal pregnancy is an extremely rare form of ectopic pregnancy, mostly occurring secondarily after tubal rupture or abortion with secondary implantation anywhere in the peritoneal cavity. Massive intra-abdominal hemorrhage is a life threatening complication associated with secondary abdominal pregnancy. Various methods and techniques have been reported in the literature for controlling hemorrhage. Here, we report a case of massive intraperitoneal haemorrhage following placental removal controlled by abdominal packing and review the literature for diagnostic and management challenges.**

Keywords: Abdominal pregnancy, abdominal packing, intraperitoneal packing

1. Introduction

Abdominal pregnancy is a rare form ectopic pregnancy occurring in 1 in 10,000 live births to 1 in 30,000 (*1*). It could be either primary or secondary with the latter being more common. Secondary abdominal pregnancy occurs following a tubal abortion or rupture with secondary implantation in the peritoneal cavity. Primary ectopic pregnancy is diagnosed with studdiford criteria which specifies that both tubes and ovaries should be normal with no tuboperitoneal fistula and an early pregnancy implanted on peritoneal surface which makes the possibility of secondary abdominal pregnancy a rare possibility (*2*). The main complication of ectopic pregnancy is life threatening hemorrhage. Here, we discuss a case of secondary abdominal pregnancy with profuse intraperitoneal haemorrhage.

2. Case Report

A 28 year lady G2P1LO presented to emergency room with ammenorrhoea for 3 months, lower abdomen pain and bleeding per vaginum for past 7 hours. On examination patient was pale, pulse 120/min, thready and hypovolemic, blood pressure was 90/60 mm Hg. On abdominal examination, there was distention, rigidity and rebound tenderness. On vaginal examination the uterus was normal in size with fullness in all fornices and tenderness in right adenexa. No adnexal lump was present. Urine pregnancy test was positive. Transvaginal Ultrasonography (Figure 1) showed a sac in right adnexa consisting of a 12 weeks old fetus with positive cardiac activity and a collection in the pelvis and abdomen. An empty uterus was seen separately. She was taken up for immediate laparotomy as a suspected case of ruptured ectopic pregnancy in view of her hemodynamic instability. On laparotomy, there was haemoperitoneum, approximately 1.7 litres of blood was drained from abdominal cavity with a live pregnancy within a gestational sac lying in the abdominal cavity. The placenta was found attached to the posterior surface of uterus and posterior leaf of right broad ligament and the peritoneum overlying the ureter (Figure 2). The right tube was distorted, enlarged, edematous and hyperemic with a hole in the mesosalpinx. Right salpingectomy was done as the right tube was distorted and damaged, to prevent recurrence. The placenta was partially separated and its complete removal triggered profuse bleeding from the placental bed ,with very friable tissue overlying the peritoneum covering the ureter. Hemostatic sutures were taken to control bleeding from posterior surface of the broad ligament. However bleeding from the peritoneum overlying the ureter continued and a decision to pack the area with ribbon gauze was taken. The packing was limited to posterior surface of uterus and pouch of Douglas. One end of the ribbon gauze was taken out abdominally through the abdominal wound. An abdominal drain was inserted. The opposite fallopian tube and ovary were normal. Patient was transfused 4

**Address correspondence to:*
Dr. Shipra Kunwar, Era's lucknow Medical College, Obstetrics and Gynaecology, Lucknow-226008, India.
E-mail: shipra.kunwar@gmail.com

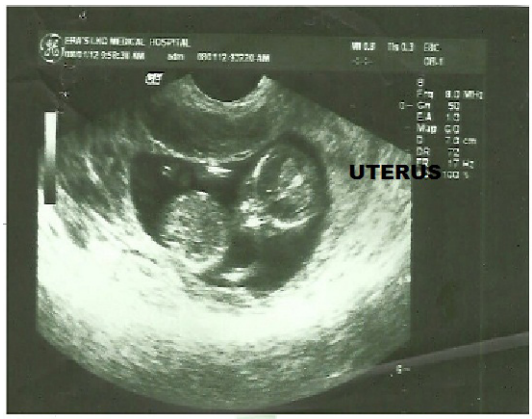


Figure 1. Ultrasound picture showing live extrauterine pregnancy.

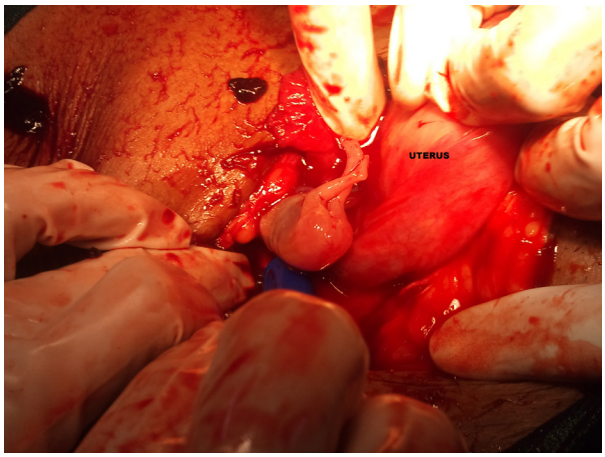


Figure 2. Per-operative finding of fetus lying in peritoneal cavity.

units of packed cells and 2 units' fresh frozen plasma and put on antibiotics.

Pack was removed after 48 hours without any complications. Abdominal drain was removed on third post-operative day. Patient improved and was discharged on sixth post-operative day after stitch removal. However, patient follow up was lost because she failed to report to the outpatient clinic after six weeks.

3. Discussion

Ectopic pregnancy has an average incidence of 1%, however, it accounts for 3-4% of pregnancy related deaths (*3*). Abdominal pregnancy is rarer and more dangerous with an incidence varying from one per 10,000 births to 1 in 30,000 (*1*). Abdominal pregnancy is difficult to diagnose and poses a serious risk of morbidity and mortality to the mother and the baby. Maternal mortality ranges between 0 and 30 percent (*4*). While the perinatal mortality ranges from 40 to 95 percent (*5*). Clinical diagnosis may be suspected from abdominal pain, vaginal bleeding, adnexal mass, gastrointestinal symptoms, tenderness and in later stages as abnormal lie, superficially palpable fetal parts, painful fetal movements, failed induction of labor and intrauterine fetal death (*6-9*).

Ultrasound and MRI are helpful in diagnosis when we have a high index of clinical suspicion. The demonstration of an empty uterus adjacent to the bladder, absence of myometrium around the fetus, unusual fetal lie, poor definition of the placenta, and relative oligohydramnios have been described on USG (*10,11*). Factors such as maternal hemodynamic status, fetal congenital abnormality, fetal viability, gestational age at presentation, and the availability of neonatal facilities should be considered while managing abdominal pregnancies (*12*). When presenting at an early gestational age the management is laparotomy for removal of the pregnancy because of risk of hemorrhage and association with a much higher incidence of congenital anomalies. With advanced pregnancy when the fetus is at the borderline limits of viability we are faced with the dilemma of continuing the pregnancy at the risk of severe hemorrhage in the mother leading to increased maternal mortality and morbidity versus terminating the pregnancy by elective, well planned laparotomy. It has been reported that if the fetus is surrounded by a normal volume of amniotic fluid, fetal outcome is good and careful wait and watch may be considered until fetal maturity (*4*). The central controversy in managing abdominal pregnancies is regarding management of the placenta and controlling hemorrhage from the placental bed following placental separation without damage to vital organs like the bowel and is the main clinical challenge (*13*). Various methods to control hemorrhage have been suggested in the literature. With a confirmed diagnosis of abdominal pregnancy giving pre-operative methotrexate to reduce blood loss at the time of surgery has been suggested (*14*). Others have preferred leaving the placenta behind (*15*). But this practice is accompanied by serious risks like secondary hemorrhage, sepsis, failure of lactation, disseminated coagulation, fistulae formation and bowel obstruction due to adhesions (*6, 16,17*).

Use of post-operative methotrexate for involution of placenta is controversial (*18*). Selective embolization of placental bed has also been reported in the literature (*19*). In our patient none of the above procedures were possible because the general condition of the patient was very low at the time of presentation, and profuse bleeding from the bed was continuing. Our institution does not have the facility for embolisation. We resorted to abdominal packing with a successful outcome. One of the main reasons for using abdominal packing in this patient was the friability of the tissue in the placental bed area overlying the ureter with risk of ureteric injury. Abdominal packing has been used effectively for uncontrolled hemorhage following caesarean hysterectomy for morbidly adherent placenta (*20*) massive hemorrhage during gynecological cancer surgery (*21*) and for post-partum hemorrhage (*22*). However, we found no case wherein it has been used to control hemorrhage in secondary abdominal pregnancy. Various

types of packs have been described in the literature with the earliest reported being the Logothetopoulos pack (*23*) others being dry laparotomy packs (*23*), transcutaneous placement of an inflated condom over a 22 fr catheter (*24*) or ribbon gauze within a penrose drain (*25*). Various complications described include a re-laparotomy for removal of pack, post-operative febrile morbidity, sepsis, small bowel obstruction, but no long term complications have been reported.

Intra-abdominal packing proved to be an effective method for controlling hemorrhage and achieving hemostasis in our case.

References

1. Badria L, Amarin Z, Jaradat A, Zahawi H, Gharaibeh A, Zobi A. Full-term viable abdominal pregnancy: A case report and review. Arch Gynecol Obstet. 2003; 268:340-342.
2. Studdiford WE. Primary peritoneal pregnancy. Am J Obstet Gynecol. 1942, 44:487-491.
3. Berg CJ, Callaghan WM, Syverson C, Henderson Z. Pregnancy-related mortality in the United States, 1998 to 2005. Obstet Gynecol. 2010; 116:1302-1309.
4. Martin JN Jr, McCaul JF 4th. Emergent management of abdominal pregnancy. Clin Obstet Gynecol. 1990; 33:438-447.
5. Ang LP, Tan AC, Yeo SH. Abdominal pregnancy: A case report and literature review. Singapore Med J. 2000; 41:454-457.
6. Rahman MS. Al-Suleiman SA, Rahman J, Al-Sibai MH. Advanced abdominal pregnancy – observations in 10 cases. Obstet Gynecol. 1982; 59:366-372.
7. Kun KY, Wong PY, Ho MW, Tai CM, Ng TK. Abdominal pregnancy presenting as a missed abortion at 16 weeks' gestation. Hong Kong Med J. 2000; 6:425-427.
8. King M, Bewes PC, Cairns J, Thornton J. Primary Surgery. Volume 1: Chapter 8 nontrauma. On-line edition available at *http://www.meb.uni-bonn.de/dtc/primsurg/docbook/html/ x5173.html accessed on 4/5/2014*. (accessed December 23, 2014)
9. Lamina MA, Akinyemi BO, Fakoya TA. Shorunmu TO, Oladapo OT. Abdominal pregnancy: A cause of failed induction of labour. Niger J Med. 2005; 14:213-217.
10. Varma R, Mascarenhas L, James D. Successful outcome of advanced abdominal pregnancy with exclusive omental insertion. Ultrasound Obstet Gynecol. 2003; 21:192-194.
11. Graham D, Johnson TR Jr, Sanders RC. Sonographic findings in abdominal pregnancy. J Ultrasound Med. 1982; 1:71-74.
12. Shafi SM, Malla MA, Salaam PA, Kirmani OS. Abdominal pregnancy as a cause of hemoperitoneum. J Emerg Trauma Shock. 2009; 2:196-198.
13. Nkusu Nunyalulendho D, Einterz EM. Advanced abdominal pregnancy: Case report and review of 163 cases reported since 1946. Rural Remote Health. 2008; 8:1087.
14. Gupta P, Sehgal A, Huria A, Mehra R. Secondary abdominal pregnancy and its associated diagnostic and operative dilemma: Three case reports. J Med Case Rep. 2009; 3:7382.
15. Hallatt JG, Grove JA. Abdominal pregnancy: A study of twenty-one consecutive cases. Am J Obstet Gynecol. 1985; 152:444-449.
16. Dahab AA, Aburass R, Shawkat W, Babgi R, Essa O, Mujallid RH. Full-term extrauterine abdominal pregnancy: A case report. J Med Case Rep. 2011; 5:531.
17. Pieh-Holder KL, Scardo JA, Costello DH. Lactogenesis failure following successful delivery of advanced abdominal pregnancy. Breastfeed Med. 2012; 7:543-546.
18. Kivikoski AI, Martin C, Weyman P, Picus D, Giudice L. Angiographic arterial embolization to control hemorrhage in abdominal pregnancy: A case report. Obstet Gynecol. 1988; 71:456-459.
19. Gary Cunningham F, Leveno KJ, Bloom SL, Hauth JC, Rouse D, Spong CY. Williams Obstetrics, 23rd ed., McGraw-Hill Professional, New York, USA, 2009.
20. Ghourab S, Al-Nuaim L, Al-Jabari A, Al-Meshari A, Mustafa MS, Abotalib Z, Al-Salman M. Abdomino-pelvic packing to control severe haemorrhage following caesarean hysterectomy. J Obstet Gynaecol. 1999; 19:155-158.
21. Finan MA, Fiorica JV, Hoffman MS, Barton DP, Gleeson N, Roberts WS, Cavanagh D. Massive pelvic hemorrhage during gynecologic cancer surgery: "Pack and go back". Gynecol Oncol. 1996; 62:390-395.
22. Robie GF, Morgan MA, Payne GG Jr, Wasemiller-Smith L. Logothetopulos pack for the management of uncontrollable postpartum hemorrhage. Am J Perinatol. 1990; 7:327-328.
23. Logothetopoulos K. Eine absolut sichere blutstillungs-methode bei vaginalen undabdominalen gynakologischen operationen. [An absolutely certain method of stopping bleeding during abdominal and vaginal operations]. Zentralbl Gynakol. 1926; 50:3202-3204.
24. Luijendijk RW, IJzermans JN, Jeekel J, Bruining HA. An inflated condom as a packing device for control of haemorrhage. Br J Surg. 1994; 81:270.
25. Awonuga AO, Merhi ZO, Khulpateea N. Abdominal packing for intractable obstetrical and gynecologic hemorrhage. Int J Gynaecol Obstet. 2006; 93:160-163.

(Received February 21, 2015; Revised March 9, 2015; Accepted March 30, 2015)

Case Report

DOI: 10.5582/irdr.2015.01012

Focal mid-ventricular anterior ballooning: An unusual pattern of Takotsubo cardiomyopathy

Selami Demirelli[1,*], Emrah Ermis[1], Engin Hatem[1], Abdulkadir Uslu[2], Lutfu Askin[3]

[1] *Department of Cardiology, Erzurum Education and Research Hospital, Erzurum, Turkey;*
[2] *Department of Cardiology, Ahi Evren Chest and Cardiovascular Surgery Education and Research Hospital, Trabzon, Turkey;*
[3] *Palandöken State Hospital, Erzurum, Turkey.*

Summary **Takotsubo cardiomyopathy (TC) or left ventricular apical ballooning syndrome is typically characterized by reversible systolic dysfunction of the apical- and mid-segments of the left ventricle. Symptoms are precipitated by intense emotional or physical stress, in the absence of obstructive coronary artery lesions. The most common presentation of TC is a transient left ventricular apical ballooning. However, recent case reports have described various patterns of TC associated with distinct regional left ventricular wall motion abnormalities. One of very rare these variants, referred to as a "mid-ventricular" type, is characterized by akinesis with or without ballooning of the mid-ventricular segment, together with a hyperdynamic base and apex. Using left ventriculography we describe an atypical form of TC with transient, focal mid-ventricular ballooning of the anterior segment, followed by complete resolution of ballooning, as observed by cardiac magnetic resonance (CMR) imaging.**

Keywords: Atypical form of takotsubo cardiomyopathy, mid-ventricular anterior ballooning, diagnosis

1. Introduction

Takotsubo cardiomyopathy (TC) was first described by Japanese physicians in 1991 (*1*). It is generally characterized by transient systolic dysfunction precipitated by a stressful event, in the absence of obstructive coronary artery lesions. For this reason TC is also known as "stress-induced" or "broken-heart" syndrome. Diagnoses of TC are becoming more common, likely due to an increase in the awareness of interactions between critical brain activity and the heart. The most common presentation of TC is an apical ballooning pattern; however, several recent case reports have described variants of TC that present with differing patterns (*2-4*). These atypical forms are associated with distinct regional left ventricular wall motion abnormalities. Left ventriculography and CMR imaging is uniquely suited for the evaluation of patients with TC and its variants. Using these imaging techniques we present a case of atypical TC that fulfills all current diagnostic criteria for TC, excluding the classical contractile LV pattern.

2. Case Report

A 59-year-old female patient with a history of hypertension and dyslipidemia was admitted to the emergency department with retrosternal chest pain after learning of her son's death. Initial physical examination of the patient was normal. Arterial blood pressure was 130/80 mmHg with a heart rate of 80 beats per minute. Her first electrocardiogram (ECG) recording in the emergency room demonstrated normal sinus rhythm with T-wave inversion on leads V1-V4. Laboratory findings indicated a significantly elevated serum level of troponin I and CKMB (troponin I, 2.44 mcg/L and CKMB, 57.1 U/L, normal values < 0.1 mcg/L and < 25 U/L, respectively). Transthoracic echocardiography identified a regional wall motion abnormality. Akinesia was detected in the mid-anterior segment, with a 45% left ventricular ejection fraction. Due to the clinical presentation of symptoms an emergency coronary angiography was performed on the patient. Cardiac

**Address correspondence to:*
Dr. Selami Demirelli, Department of Cardiology, Erzurum Education and Research Hospital, Erzurum, Turkey.
E-mail: demirelli23@yahoo.com

catheterization and angiography revealed no significant coronary artery disease and coronary artery spasm provocation tests were negative. Left ventriculography demonstrated akinesis of the mid-ventricular anterior segment with ballooning and a hyperdynamic base and apex (Figure 1). The patient was treated with aspirin, statins, beta-blockers and angiotensin-converting enzyme inhibitor and was discharged in stable condition on the third day. Four weeks following discharge CMR imaging was performed on the patient. We found a complete lack of ballooning together with an absence of scaring and no abnormalities with regards to wall motion (Figure 2).

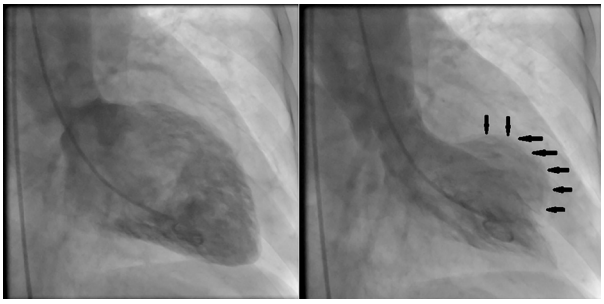


Figure 1. Left ventriculography demonstrated akinesis of the mid-ventricular anterior segment with ballooning and a hyperdynamic base and apex.

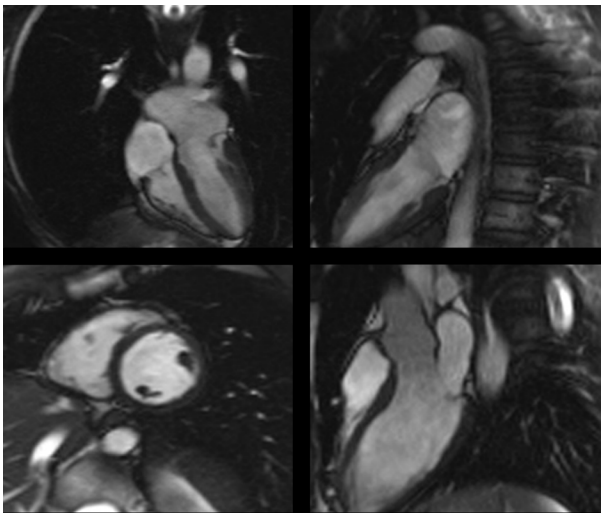


Figure 2. After four weeks cardiac magnetic resonance imaging showed complete lack of ballooning together with an absence of scaring and no abnormalities with regards to wall motion.

3. Discussion

Takotsubo cardiomyopathy is typically characterized by reversible systolic dysfunction of the apical and mid-segments of the left ventricle, in the absence of obstructive coronary artery lesions. Clinical symptoms of TC mimic those of acute coronary syndrome (ACS), the most common of which are chest pain and dyspnea. The patient may also present with hypotension secondary to decreased stroke volume. Cardiac arrest, syncope and arrhythmias, with or without a prolonged QT interval, have also been described (*5,6*). Transient heart failure is the most common clinical complication, although the prognosis is generally favorable despite limited published data chronicling long-term patient outcome.

The majority of TC patients present with dynamic ECG changes and precordial ST segment elevation or diffuse T-wave inversions. These changes are typically associated with a modest rise in the level of serum cardiac enzymes. Wall motion abnormalities and functional mitral regurgitation are evaluated in patents using echocardiography, whereas coronary angiography is considered the gold standard to differentiate TC from ACS. The pathophysiology of TC is currently unclear; however, several theories have been proposed recently. A role for catecholamines in triggering TC appears feasible as high levels of circulating catecholamine can cause cardiotoxicity and coronary artery spasms, resulting in myocardial stunning (*7,8*). Low estrogen levels have also been implicated. It is known that estrogen provides cardioprotection in premenopausal females by downregulating beta-1 adrenoceptors. Accordingly, estrogen supplementation might protect postmenopausal females against the development of TC (*8*).

Diagnosis criteria for TC, as proposed by the Mayo Clinic, relate primarily to wall motion abnormalities, such as transient hypokinesis, akinesis, or dyskinesis of the LV mid segments, with or without apical involvement. These regional wall motion abnormalities extend beyond a single epicardial vascular distribution (*9*). Several novel variants of TC have been described recently; however, "typical" TC remains the pattern observed most frequently. Atypical forms are associated with distinct regional left ventricular wall motion abnormalities, although reverse or inverted TC, midventricular type and biventricular (right and left ventricular) ballooning group have also been described (*2-4*). In reverse or inverted TC, the apex is hyperdynamic and the base is akinetic (*2*). Mid-ventricular type is characterized by akinesis with or without ballooning of the mid-ventricular segment together with a hyperdynamic base and apex (*3*). Other variants described include akinesis of various left ventricle and right ventricle segments (*4*). In this case report we describe an atypical form of TC with transient, focal mid-ventricular anterior-segment ballooning.

Our report raises the intriguing question of why apical segments are predisposed to stunning, compared to the base, in patients with TC. Several potential explanations can be considered. First, the apex may be more responsive to adrenergic stimulation or demonstrate an increased density of catecholamine-sensitive receptors. Second, the apex is structurally vulnerable. It possesses a relatively limited elasticity

reserve and can easily become ischemic as a consequence of limited coronary vasculature (*10*). Interestingly, a recent case report identified different variants of typical and atypical forms in the same patient (*11*). Variation between individuals with regard to the local release of catecholamines or in the distribution of autonomic innervation of the LV wall may therefore contribute to the observation of different variants.

Coronary angiography is considered the gold standard for the diagnosis of TC. Left ventriculography is used to identify abnormalities in wall motion and is therefore key in differentiating the variants. CMR imaging is uniquely suited for the evaluation of patients with TC. Critically, it provides markers to identify reversible versus irreversible injury, which are important criteria when confirming a diagnosis of TC and excluding similar acute cardiac diseases, superficially ACS or myocarditis (*12*). In our case CMR imaging was utilized to demonstrate the reversibility of systolic LV dysfunction after the acute phase.

4. Conclusion

Several novel variants of TC have been described in recent case reports. Our case illustrates an unusual presentation of takotsubo cardiomyopathy. Coronary angiography and left ventriculography are considered the gold standard for the diagnosis of TC and its differentiation from ACS. Together with CMR imaging these methods are also effective at distinguishing between typical and atypical forms of TC. The pathophysiological mechanisms driving distinct LV wall motion abnormalities in different variants of TC remain unclear. Therefore, additional studies are warranted to investigate the role of genetic susceptibility and patient predisposition to physical and emotional stress in development of this disease.

Acknowledgements

A summary of this study were presented as an oral presentation at the 64th ESCVS & 11th International Congress of Update in Cardiology and Cardiovascular Surgery, and the abstract was published in the American Journal of Cardiology, Volume 115, Supplement 1, March 16, Pages S126-S127.

References

1. Dote K, Sato H, Tateishi H, Uchida T, Ishihara M. Myocardial stunning due to simultaneous multivessel coronaryspasms: A review of 5 cases. J Cardiol. 1991; 21:203-214.
2. Caccioti L, Camastra GS, Musarò S, Proietti I, Semeraro R, Martina C, Lupparelli F, Ansalone G. Stress cardiomyopathy: Transient basal ballooning. J Cardiovasc Med (Hagerstown). 2010; 11:764-767.
3. Kurowski V, Kaiser A, von Hof K, Killermann DP, Mayer B, Hartmann F, Chunkert H, Radke PW. Apical and midventricular transient left ventricular dysfunction syndrome (tako-tsubo cardiomyopathy): Frequency, mechanisms, and prognosis. Chest. 2007; 132:809-816.
4. Eitel I, Schuler G, Gutberlet M, Thiele H. Biventricular stress-induced (takotsubo) cardiomyopathy with left midventricular and right apical ballooning. Int J Cardiol. 2011; 151:e63-e64.
5. Lee YP, Poh KK, Lee CH, Tan HC, Razak A, Chia BL, Low AF. Diverse clinical spectrum of stress-induced cardiomyopathy. Int J Cardiol. 2009; 133:272-275.
6. Pant S, Deshmukh A, Mehta K, Badheka AO, Tuliani T, Patel NJ, Dabhadkar K, Prasad A, Paydak H. Burden of arrhythmias in patients with Takotsubo cardiomyopathy (apical ballooning syndrome). Int J Cardiol. 2013; 170:64-68.
7. Balkin DM, Cohen LS. Takotsubo Syndrome. Coron Artery Dis. 2011; 22:206-214.
8. Jabara R, Gadesam R, Pendyala L, Chronos N, King SB, Chen JP. Comparison of the clinical characteristics of apical and non-apical variants of "broken heart" (takotsubo) syndrome in the United States. J Invasive Cardiol. 2009; 21:216-222.
9. Scantlebury DC, Prasad A. Diagnosis of Takotsubo cardiomyopathy. Circ J. 2014; 78:2129-2139.
10. Mori H, Ishikawa S, Kojima S, Hayashi J, Watanabe Y, Hoffman JI, Okino H. Increased responsiveness of left ventricular apical myocardium to adrenergic stimuli. Cardiovasc Res. 1993; 27:192-198.
11. Yoshida T, Nishizawa T, Yajima K, Tsuruoka M, Fujimaki T, Oguri M, Kato K, Hibino T, Ohte N, Yokoi K, Kimura G. A rare case of tako-tusbo cardiomyopathy with variable forms of left ventricular dysfunction: A new entity. Int J Cardiol. 2009; 134:e73-e75.
12. Eitel I, von Knobelsdorff-Brenkenhoff F, Bernhardt P, Carbone I, Muellerleile K, Aldrovandi A, Francone M, Desch S, Gutberlet M, Strohm O, Schuler G, Schulz-Menger J, Thiele H, Friedrich MG. Clinical characteristics and cardiovascular magnetic resonance findings in stress (takotsubo) cardiomyopathy. JAMA. 2011; 306:277-286.

(Received April 2, 2015; Accepted April 16, 2015)

Letter

DOI: 10.5582/irdr.2015.01003

Can Hutchinson-Gilford progeria syndrome be cured in the future?

Neeha Abdul Rehman*, Aneeqa Abdul Rehman, Isra Najib Ashraf, Shahrukh Ahmed

Dow Medical College, Karachi, Pakistan.

Summary **Progeria is a rare genetic disease that manifests with progressive symptoms eventually leading to death. The only current treatment protocol of such patients is symptom based. However, recent trials are testing potential and promising drugs to treat the underlying genetic mutation and increase life expectancy of such patients.**

Keywords: Progeria, Cure, farnesyltransferase inhibitors

Progeria or Hutchinson-Gilford progeria syndrome (HGPS) is a very rare and fatal premature genetic disease belonging to the group of progeroid syndromes. Having an incidence of 1 per 8 million live births, the disease manifests with symptoms of accelerated aging among children, eventually leading to death at an early age. HGPS is caused by mutation of the *LMNA* gene that produces farnesylated abnormal Lamin A protein, Progerin. Physiologically, *LMNA* gene encodes for a pre-structural protein prelamin A that provisionally attaches to the nuclear rim on farnesylation with a functional group. Once attached, the farnesyl group is eliminated from prelamin A forming the protein Lamin A which in turn forms the nuclear lamina. However in HGPS, a point mutation of *LMNA* gene causes the fanesyl functional group to become permanently attached to prelamin A preventing further modification to Lamin A. Accumulation of "Progerin" on the nuclear rim disintegrates structural support of nuclear lamina and disorganizes nuclear processes like DNA and RNA synthesis (*1*). No specific treatment is available so far with management aiming only to control complications (especially cardiovascular problems).

Current preclinical studies have revealed quite promising results for farnesyltransferase inhibitors (FTIs) and other potential drugs for treatment of this disease. Initially developed as a target drug for an oncogenic *RAS* gene, FTIs have shown to block the enzyme responsible for the farnesylation step on prelamin A in HGPS children. Although not curing the pathological cause, inhibition of this step averts buildup of Progerin on the nuclear rim preventing further progress of the disease among children (*2*). Frequency of clinical strokes, headaches, and other complications were greatly reduced improving the lifespan of children in a trial (*2*). In another trial, Wang *et al*. demonstrated reversing nucleus abnormalities in HGPS transgenic mice using FTIs or statins (Pravastatin and Zoledronate) by blocking farnesylation. Also, positive outcomes showing reversal of the disease for *in-vivo* cultured cells derived from humans were noted in the study (*3*). Ibrahim *et al*. recent preclinical study has further helped us find another solution for HGPS. By inhibiting isoprenylcysteine methylation (responsible for accumulation of Progerin), normal cellular processes were reported in transgenic mice and human cells (*4*). However, inhibition only prevented further progression of HGPS.

Although there is still no approved drug for treatment of HGPS by the Food and Drug Administration, these trials are offering a glimpse of something big for HGPS treatment in the future. There is a long way to finding a cure but there is no doubt that such outcomes have a massive potential of helping us find one.

Released online in J-STAGE as advance publication April 7, 2015.

**Address correspondence to:*
Dr. Neeha Abdul Rehman, Dow Medical College, Baba-e-Urdu Road, Karachi 74400, Pakistan.
E-mail: neeha171@gmail.com

References

1. Cao K, Graziotto JJ, Blair CD, Mazzulli JR, Erdos MR, Krainc D, Collins FS. Rapamycin reverses cellular phenotypes and enhances mutant protein clearance in Hutchinson-Gilford progeria syndrome cells. Sci Transl Med. 2011; 3:89ra58.
2. Ullrich NJ, Kieran MW, Miller DT, Gordon LB, Cho YJ,

Silvera VM, Giobbie-Hurder A, Neuberg D, Kleinman ME. Neurologic features of Hutchinson-Gilford progeria syndrome after lonafarnib treatment. Neurology. 2013; 81:427-430.
3. Wang Y, Ostlund C, Worman HJ. Blocking protein farnesylation improves nuclear shape abnormalities in keratinocytes of mice expressing the prelamin A variant in Hutchinson-Gilford progeria syndrome. Nucleus. 2010; 1:432-439.
4. Ibrahim MX, Sayin VI, Akula MK, Liu M, Fong LG, Young SG, Bergo MO. Targeting isoprenylcysteine methylation ameliorates disease in a mouse model of progeria. Science. 2013; 340:1330-1333.

(Received February 15, 2015; Revised March 8, 2015; Accepted March 30, 2015)

IRDR
Intractable & Rare Diseases Research

Guide for Authors

1. Scope of Articles

Intractable & Rare Diseases Research is an international peer-reviewed journal. Intractable & Rare Diseases Research devotes to publishing the latest and most significant research in intractable and rare diseases. Articles cover all aspects of intractable and rare diseases research such as molecular biology, genetics, clinical diagnosis, prevention and treatment, epidemiology, health economics, health management, medical care system, and social science in order to encourage cooperation and exchange among scientists and clinical researchers.

2. Submission Types

Original Articles should be well-documented, novel, and significant to the field as a whole. An Original Article should be arranged into the following sections: Title page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgments, and References. Original articles should not exceed 5,000 words in length (excluding references) and should be limited to a maximum of 50 references. Articles may contain a maximum of 10 figures and/or tables.

Brief Reports definitively documenting either experimental results or informative clinical observations will be considered for publication in this category. Brief Reports are not intended for publication of incomplete or preliminary findings. Brief Reports should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 4 figures and/or tables and 30 references. A Brief Report contains the same sections as an Original Article, but the Results and Discussion sections should be combined.

Reviews should present a full and up-to-date account of recent developments within an area of research. Normally, reviews should not exceed 8,000 words in length (excluding references) and should be limited to a maximum of 100 references. Mini reviews are also accepted.

Policy Forum articles discuss research and policy issues in areas related to life science such as public health, the medical care system, and social science and may address governmental issues at district, national, and international levels of discourse. Policy Forum articles should not exceed 2,000 words in length (excluding references).

Case Reports should be detailed reports of the symptoms, signs, diagnosis, treatment, and follow-up of an individual patient. Case reports may contain a demographic profile of the patient but usually describe an unusual or novel occurrence. Unreported or unusual side effects or adverse interactions involving medications will also be considered. Case Reports should not exceed 3,000 words in length (excluding references).

News articles should report the latest events in health sciences and medical research from around the world. News should not exceed 500 words in length.

Letters should present considered opinions in response to articles published in Intractable & Rare Diseases Research in the last 6 months or issues of general interest. Letters should not exceed 800 words in length and may contain a maximum of 10 references.

3. Editorial Policies

Ethics: Intractable & Rare Diseases Research requires that authors of reports of investigations in humans or animals indicate that those studies were formally approved by a relevant ethics committee or review board.

Conflict of Interest: All authors are required to disclose any actual or potential conflict of interest including financial interests or relationships with other people or organizations that might raise questions of bias in the work reported. If no conflict of interest exists for each author, please state "There is no conflict of interest to disclose".

Submission Declaration: When a manuscript is considered for submission to Intractable & Rare Diseases Research, the authors should confirm that 1) no part of this manuscript is currently under consideration for publication elsewhere; 2) this manuscript does not contain the same information in whole or in part as manuscripts that have been published, accepted, or are under review elsewhere, except in the form of an abstract, a letter to the editor, or part of a published lecture or academic thesis; 3) authorization for publication has been obtained from the authors' employer or institution; and 4) all contributing authors have agreed to submit this manuscript.

Cover Letter: The manuscript must be accompanied by a cover letter signed by the corresponding author on behalf of all authors. The letter should indicate the basic findings of the work and their significance. The letter should also include a statement affirming that all authors concur with the submission and that the material submitted for publication has not been published previously or is not under consideration for publication elsewhere. The cover letter should be submitted in PDF format. For example of Cover Letter, please visit http://www.irdrjournal.com/downcentre.php (Download Centre).

Copyright: A signed JOURNAL PUBLISHING AGREEMENT (JPA) form must be provided by post, fax, or as a scanned file before acceptance of the article. Only forms with a hand-written signature are accepted. This copyright will ensure the widest possible dissemination of information. A form facilitating transfer of copyright can be downloaded by clicking the appropriate link and can be returned to the e-mail address or fax number noted on the form (Please visit Download Centre). Please note that your manuscript will not proceed to the next step in publication until the JPA Form is received. In addition, if excerpts from other copyrighted works are included, the author(s) must obtain written permission from the copyright owners and credit the source(s) in the article.

Suggested Reviewers: A list of up to 3 reviewers who are qualified to assess the scientific merit of the study is welcomed. Reviewer information including names, affiliations, addresses, and e-mail should be provided at the same time the manuscript is submitted online. Please do not suggest reviewers with known conflicts of interest, including participants or anyone with a stake in the proposed research; anyone from the same institution; former students, advisors, or research collaborators (within the last three years); or close personal contacts. Please note that the Editor-in-Chief may accept one or more of the proposed reviewers or may request a review by other qualified persons.

Language Editing: Manuscripts prepared by authors whose native language is not English should have their work proofread by a native English speaker before submission. If not, this might delay the publication of your manuscript in Intractable & Rare Diseases Research.

The Editing Support Organization can provide English proofreading, Japanese-English translation, and Chinese-English translation services to authors who want to publish in Intractable & Rare Diseases Research and need assistance before submitting a manuscript. Authors can visit this organization directly at http://www.iacmhr.com/iac-eso/support.php?lang=en. IAC-ESO was established to facilitate manuscript preparation by researchers whose native language is not English and to help edit works intended for international academic journals.

4. Manuscript Preparation

Manuscripts should be written in clear, grammatically correct English and submitted as a Microsoft Word file in a single-column format. Manuscripts must be paginated and typed in 12-point Times New Roman font with 24-point line spacing. Please do not embed figures in the text. Abbreviations should be used as little as possible and should be explained at first mention unless the term is a well-known abbreviation (*e.g.* DNA). Single words should not be abbreviated.

Title page: The title page must include 1) the title of the paper (Please note the title should be short, informative, and contain the major key words); 2) full name(s) and affiliation(s) of the author(s), 3) abbreviated names of the author(s), 4) full name, mailing address, telephone/fax numbers, and e-mail address of the corresponding author; and 5) conflicts of interest (if you have an actual or potential conflict of interest to disclose, it must be included as a footnote on the title page of the

manuscript; if no conflict of interest exists for each author, please state "There is no conflict of interest to disclose"). Please visit Download Centre and refer to the title page of the manuscript sample.

Abstract: The abstract should briefly state the purpose of the study, methods, main findings, and conclusions. For article types including Original Article, Brief Report, Review, Policy Forum, and Case Report, a one-paragraph abstract consisting of no more than 250 words must be included in the manuscript. For News and Letters, a brief summary of main content in 150 words or fewer should be included in the manuscript. Abbreviations must be kept to a minimum and non-standard abbreviations explained in brackets at first mention. References should be avoided in the abstract. Key words or phrases that do not occur in the title should be included in the Abstract page.

Introduction: The introduction should be a concise statement of the basis for the study and its scientific context.

Materials and Methods: The description should be brief but with sufficient detail to enable others to reproduce the experiments. Procedures that have been published previously should not be described in detail but appropriate references should simply be cited. Only new and significant modifications of previously published procedures require complete description. Names of products and manufacturers with their locations (city and state/country) should be given and sources of animals and cell lines should always be indicated. All clinical investigations must have been conducted in accordance with Declaration of Helsinki principles. All human and animal studies must have been approved by the appropriate institutional review board(s) and a specific declaration of approval must be made within this section.

Results: The description of the experimental results should be succinct but in sufficient detail to allow the experiments to be analyzed and interpreted by an independent reader. If necessary, subheadings may be used for an orderly presentation. All figures and tables must be referred to in the text.

Discussion: The data should be interpreted concisely without repeating material already presented in the Results section. Speculation is permissible, but it must be well-founded, and discussion of the wider implications of the findings is encouraged. Conclusions derived from the study should be included in this section.

Acknowledgments: All funding sources should be credited in the Acknowledgments section. In addition, people who contributed to the work but who do not meet the criteria for authors should be listed along with their contributions.

References: References should be numbered in the order in which they appear in the text. Citing of unpublished results, personal communications, conference abstracts, and theses in the reference list is not recommended but these sources may be mentioned in the text. In the reference list, cite the names of all authors when there are fifteen or fewer authors; if there are sixteen or more authors, list the first three followed by *et al*. Names of journals should be abbreviated in the style used in PubMed. Authors are responsible for the accuracy of the references. Examples are given below:

Example 1 (Sample journal reference):
Inagaki Y, Tang W, Zhang L, Du GH, Xu WF, Kokudo N. Novel aminopeptidase N (APN/CD13) inhibitor 24F can suppress invasion of hepatocellular carcinoma cells as well as angiogenesis. Biosci Trends. 2010; 4:56-60.

Example 2 (Sample journal reference with more than 15 authors):
Darby S, Hill D, Auvinen A, *et al*. Radon in homes and risk of lung cancer: Collaborative analysis of individual data from 13 European case-control studies. BMJ. 2005; 330:223.

Example 3 (Sample book reference):
Shalev AY. Post-traumatic stress disorder: Diagnosis, history and life course. In: Post-traumatic Stress Disorder, Diagnosis, Management and Treatment (Nutt DJ, Davidson JR, Zohar J, eds.). Martin Dunitz, London, UK, 2000; pp. 1-15.

Example 4 (Sample web page reference):
World Health Organization. The World Health Report 2008 – primary health care: Now more than ever. *http://www.who.int/whr/2008/whr08_en.pdf* (accessed September 23, 2010).

Tables: All tables should be prepared in Microsoft Word or Excel and should be arranged at the end of the manuscript after the References section. Please note that tables should not in image format. All tables should have a concise title and should be numbered consecutively with Arabic numerals. If necessary, additional information should be given below the table.

Figure Legend: The figure legend should be typed on a separate page of the main manuscript and should include a short title and explanation. The legend should be concise but comprehensive and should be understood without referring to the text. Symbols used in figures must be explained.

Figure Preparation: All figures should be clear and cited in numerical order in the text. Figures must fit a one- or two-column format on the journal page: 8.3 cm (3.3 in.) wide for a single column, 17.3 cm (6.8 in.) wide for a double column; maximum height: 24.0 cm (9.5 in.). Please make sure that artwork files are in an acceptable format (TIFF or JPEG) at minimum resolution (600 dpi for illustrations, graphs, and annotated artwork, and 300 dpi for micrographs and photographs). Please provide all figures as separate files. Please note that low-resolution images are one of the leading causes of article resubmission and schedule delays. All color figures will be reproduced in full color in the online edition of the journal at no cost to authors.

Units and Symbols: Units and symbols conforming to the International System of Units (SI) should be used for physicochemical quantities. Solidus notation (*e.g.* mg/kg, mg/mL, mol/mm^2/min) should be used. Please refer to the SI Guide www.bipm.org/en/si/ for standard units.

Supplemental data: Supplemental data might be useful for supporting and enhancing your scientific research and Intractable & Rare Diseases Research accepts the submission of these materials which will be only published online alongside the electronic version of your article. Supplemental files (figures, tables, and other text materials) should be prepared according to the above guidelines, numbered in Arabic numerals (*e.g.*, Figure S1, Figure S2, and Table S1, Table S2) and referred to in the text. All figures and tables should have titles and legends. All figure legends, tables and supplemental text materials should be placed at the end of the paper. Please note all of these supplemental data should be provided at the time of initial submission and note that the editors reserve the right to limit the size and length of Supplemental Data.

5. Submission Checklist

The Submission Checklist will be useful during the final checking of a manuscript prior to sending it to Intractable & Rare Diseases Research for review. Please visit Download Centre and download the Submission Checklist file.

6. Online submission

Manuscripts should be submitted to Intractable & Rare Diseases Research online at http://www.irdrjournal.com. The manuscript file should be smaller than 5 MB in size. If for any reason you are unable to submit a file online, please contact the Editorial Office by e-mail at office@irdrjournal.com

7. Accepted manuscripts

Proofs: Galley proofs in PDF format will be sent to the corresponding author *via* e-mail. Corrections must be returned to the editor (office@irdrjournal.com) within 3 working days.

Offprints: Authors will be provided with electronic offprints of their article. Paper offprints can be ordered at prices quoted on the order form that accompanies the proofs.

Page Charge: No page charges will be levied to authord for the publication of their article except for reprints.

(As of February 2013)

Editorial and Head Office:

Pearl City Koishikawa 603
2-4-5 Kasuga, Bunkyo-ku
Tokyo 112-0003, Japan
Tel: +81-3-5840-9968
Fax: +81-3-5840-9969
E-mail: office@irdrjournal.com

IRDR
Intractable & Rare Diseases Research

JOURNAL PUBLISHING AGREEMENT (JPA)

Manuscript No.:

Title:

Corresponding Author:

The International Advancement Center for Medicine & Health Research Co., Ltd. (IACMHR Co., Ltd.) is pleased to accept the above article for publication in Intractable & Rare Diseases Research. The International Research and Cooperation Association for Bio & Socio-Sciences Advancement (IRCA-BSSA) reserves all rights to the published article. Your written acceptance of this JOURNAL PUBLISHING AGREEMENT is required before the article can be published. Please read this form carefully and sign it if you agree to its terms. The signed JOURNAL PUBLISHING AGREEMENT should be sent to the Intractable & Rare Diseases Research office (Pearl City Koishikawa 603, 2-4-5 Kasuga, Bunkyo-ku, Tokyo 112-0003, Japan; E-mail: office@irdrjournal.com; Tel: +81-3-5840-9968; Fax: +81-3-5840-9969).

1. Authorship Criteria

As the corresponding author, I certify on behalf of all of the authors that:

1) The article is an original work and does not involve fraud, fabrication, or plagiarism.
2) The article has not been published previously and is not currently under consideration for publication elsewhere. If accepted by Intractable & Rare Diseases Research, the article will not be submitted for publication to any other journal.
3) The article contains no libelous or other unlawful statements and does not contain any materials that infringes upon individual privacy or proprietary rights or any statutory copyright.
4) I have obtained written permission from copyright owners for any excerpts from copyrighted works that are included and have credited the sources in my article.
5) All authors have made significant contributions to the study including the conception and design of this work, the analysis of the data, and the writing of the manuscript.
6) All authors have reviewed this manuscript and take responsibility for its content and approve its publication.
7) I have informed all of the authors of the terms of this publishing agreement and I am signing on their behalf as their agent.

2. Copyright Transfer Agreement

I hereby assign and transfer to IACMHR Co., Ltd. all exclusive rights of copyright ownership to the above work in the journal Intractable & Rare Diseases Research, including but not limited to the right 1) to publish, republish, derivate, distribute, transmit, sell, and otherwise use the work and other related material worldwide, in whole or in part, in all languages, in electronic, printed, or any other forms of media now known or hereafter developed and the right 2) to authorize or license third parties to do any of the above.

I understand that these exclusive rights will become the property of IACMHR Co., Ltd., from the date the article is accepted for publication in the journal Intractable & Rare Diseases Research. I also understand that IACMHR Co., Ltd. as a copyright owner has sole authority to license and permit reproductions of the article.

I understand that except for copyright, other proprietary rights related to the Work (*e.g.* patent or other rights to any process or procedure) shall be retained by the authors. To reproduce any text, figures, tables, or illustrations from this Work in future works of their own, the authors must obtain written permission from IACMHR Co., Ltd.; such permission cannot be unreasonably withheld by IACMHR Co., Ltd.

3. Conflict of Interest Disclosure

I confirm that all funding sources supporting the work and all institutions or people who contributed to the work but who do not meet the criteria for authors are acknowledged. I also confirm that all commercial affiliations, stock ownership, equity interests, or patent-licensing arrangements that could be considered to pose a financial conflict of interest in connection with the article have been disclosed.

Corresponding Author's Name (Signature):

Date: