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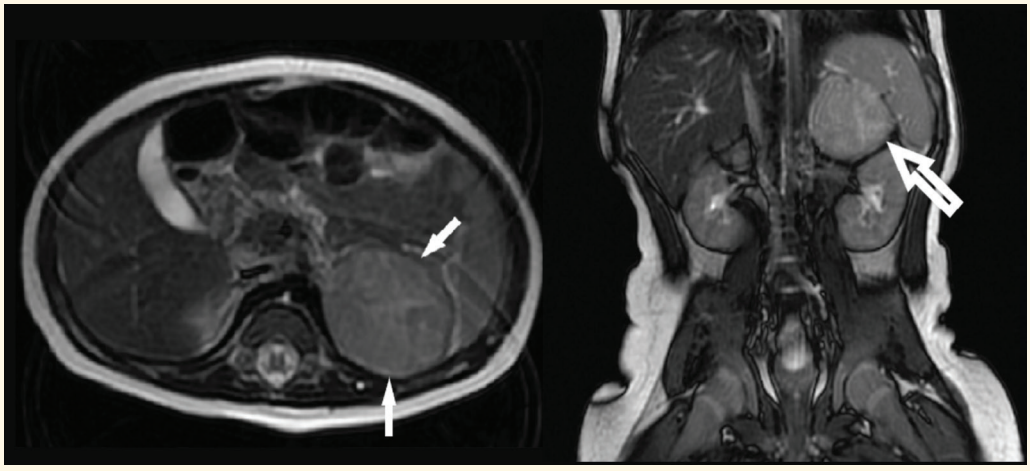

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Aims and Scope

The Journal of Pediatric Research is the official publication of Ege University and Ege Children's Foundation. It is a peer-reviewed journal published quarterly in March, June, September and December in English.

The Journal of Pediatric Research is a peer-reviewed, open-access journal, which publishes original research articles, invited review articles, clinical reports and case reports in all areas of pediatric research.

The journal publishes original research and review articles, which summarize recent developments about a particular subject based on standards of excellence and expert review and case reports. The target audience includes specialists in general pediatrics and pediatric subspecialties (Emergency Medicine, Allergy and Immunology, Endocrinology, Gastroenterology, Hepatology and Nutrition, Genetics, Cardiology, Hematology-Oncology, Infectious Diseases, Metabolism, Nephrology, Neurology, Rheumatology, Pulmonology, Social Pediatrics, Newborn, Critical Care Medicine, Ethics and Health Service Research), as well as relevant specialities such as Pediatric Surgery, Child and Adolescent Psychiatry, Pedodontics, Pediatric Nursing and Family Physicians.

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Case reports: the CARE case report guidelines

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Animal studies: ARRIVE and Guide for the Care and Use of Laboratory Animals

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and discussion sections. The whole text must not exceed 1500 words. Reviews are texts in which a current subject is examined independently, with reference to scientific literature. The whole text must not exceed 18 A4 paper sheets. Letters to the Editor must be manuscripts, which do not exceed 1000 words, with reference to scientific literature, and those written in response to issued literature or those which include development in the field of paediatrics. These manuscripts do not contain an abstract. The number of references is limited to 5.

Abbreviations: Abbreviations should be defined at first mention and used consistently thereafter. Internationally accepted abbreviations should be used; refer to scientific writing guides as necessary.

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To ensure controllability of the research findings, the study design, study sample, and the methodological approaches and applications should be explained, and their sources should be presented.

The "p" value defined as the limit of significance along with appropriate indicators of measurement error and uncertainty (confidence interval, etc.) should be specified. Statistical terms, abbreviations and symbols used in the article should be described, and the software used should be defined.

Statistical terminology (random, significant, correlation, etc.) should not be used in non-statistical contexts.

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Results: The detailed results of the study should be given, and the statistical significance level should be indicated.

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Editorial

Dear JPR Readers,

We are pleased to share with you the fourth issue of "The Journal of Pediatric Research" in 2022. The Journal of Pediatric Research is indexed in Web of Science-Emerging Sources Citation Index (ESCI), Embase, Directory of Open Access Journals (DOAJ), EBSCO, British Library, CINAHL Complete Database, ProQuest, Gale/Cengage Learning, Index Copernicus, Tübitak/Ulakbim TR Index, TurkMedline, J-GATE, IdealOnline, ROOT INDEXING, Hinari, GOALI, ARDI, OARE, AGORA, EuroPub and Türkiye Citation Index.

In this issue, we present you 13 articles including 11 original research, and two case reports from different disciplines. In this issue the readers will find the opportunity to read the article about the impact of multiple viral infection in children with severe lower respiratory tract infections which are associated with increased invasive and non-invasive support.

We would like to recommend our readers to spend time for the resveratrol supplementation in intestinal epithelial culture model. In this issue there is an article about an early predictive biomarker of steroid responsiveness in the first episode of idiopathic nephrotic syndrome.

This issue includes some different topics about newborns such as less invasive surfactant administration, dermal progression of neonatal jaundice, non-invasive measurements in the diagnosis of hyperbilirubinemia and impact of multi-strain probiotic on healthcare-associated bloodstream infection incidence.

In this issue you will find the opportunity to read article about micafungin effectiveness in treating patients with candidemia and which was found to be safe and effective. Also, there is a cross-sectional study about the assessment of the existence of liver dysfunction in Indian children with HIV infection.

We would like to acknowledge the authors, the reviewers, editorial team and Galenos Publishing House for their support in the preparation of this issue. We look forward to your scientific contributions in our future issues.

Best wishes

Ebru Canda

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Original Article

The Impact of Multiple Viral Infection in Children with Severe Lower Respiratory Tract Infections

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ABSTRACT

Aim: We aimed to compare the clinical features and outcomes between single and multiple viral pathogens in children with severe lower respiratory tract infections (LRTIs) in a pediatric intensive care unit (PICU).

Materials and Methods: This study was conducted retrospectively in patients who were admitted to a PICU between March, 2018 and March, 2020. The subjects were divided into two groups, single viral infection and multiple viral infection. The epidemiologic characteristics, clinical features, disease severity and outcomes were compared between these single and multiple viral infection groups.

Results: During this study period, positive polymerase chain reaction (PCR) tests were carried out on 136 (29%) children among the 468 children admitted to the PICU with the diagnosis of LRTI. Rhinovirus and Respiratory Syncytial Virus (RSV) were the most commonly identified viruses (44.1% and 35.2%, respectively). Two viruses were detected in thirty-nine (28.6%) of samples via PCR tests. Rhinovirus and RSV co-infection was the most common combination (10/39, 25.6%) in our cohort. The multiple viral infection group had higher PRISM scores than the single virus infection group (10 vs. 7, respectively, p=0.009). In the multiple viral infection group, the invasive ventilatory support rate (56.4% vs 36.1%, p=0.030) and the non-invasive ventilatory (NIV) support rate (43.5% vs 6.1%, p=0.018) were significantly higher than in the single viral infection group.

Conclusion: Lower respiratory multi-viral infections are associated with increased invasive and NIV support requirements. Close monitoring in a unit where support can be provided is essential for those infants with multi-viral LRTIs.

Keywords: Respiratory viruses, children, co-infection, critical care, lower respiratory tract infections

Introduction

Viral lower respiratory tract infections (LRTIs) are major causes of hospitalization for children under 2 years of age. The most frequent respiratory virus identified during hospitalization in children admitted to the pediatric intensive care unit (PICU) is Respiratory Syncytial Virus (RSV). Rhinovirus/enterovirus, Influenza A/B, Coronavirus, Parainfluenza virus, human Metapneumovirus (hMPV) and Bocaviruses are the other common viral causes of LRTIs. In recent years, improvements in molecular techniques including multiplex polymerase chain reaction (PCR) tests have allowed for the detection of viral pathogens with a wide spectrum. In children with LRTIs, PCR tests detected viral pathogens in up to 95% of cases. PCR test results are positive in up to 40% for viral co-infections (1-3).

In the literature, there are controversies regarding the relationship between viral co-infection and disease severity. In studies including children who were admitted to PICUs, viral co-infection was not associated with invasive

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mechanical ventilation requirements and/or mortality (4,5).

In this study, we aimed to compare the clinical features and outcomes between single and multiple viral pathogens in children with LRTIs in a PICU.

Materials and Methods

This study was a retrospective and comparative study of patients admitted to the PICU at Ege University Children's Hospital, a tertiary intensive care unit with 17 beds. We identified children aged between 1 month and 18 years with severe LTRI who had undergone PCR tests. From March 1st, 2018 to March 1st, 2020, 1,170 patients were admitted to the PICU and 490 (41.8%) had a primary diagnosis of severe LRTI. Four hundred and sixty-eight (40%, 468/1,170) children who had undergone PCR tests were included in this study.

Detailed clinical data on each patient were collected from secure electronic medical records. This included demographic and clinical characteristics; age, sex, gestational age, laboratory results, Pediatric Risk of Mortality Score (PRISM), radiological findings, and outcome data including; length of PICU stay, type of respiratory support, length of respiratory support, positive bacterial lower respiratory tract co-infection and mortality. Bacterial co-infection was defined as the identification of a bacterial pathogen in culture from an endotracheal specimen in those children who had an endotracheal tube. Tracheal aspirate samples were obtained from ventilated patients through an endotracheal tube by direct tracheal aspiration. Those patients who required mechanical ventilation with negative admission endotracheal aspirate sampling but tested positive after the second day of intubation were determined to be ventilatory-associated pneumonia and they were excluded from this study. Pediatric acute respiratory distress syndrome was defined according to the Pediatric Acute Lung Injury Consensus Committee criteria: namely, findings of new infiltrate(s) consistent with acute pulmonary parenchymal disease and PaO_2/FiO_2 (P/F) ratio <300 or oxygen saturation/FiO_2 (S/F) ratio ≤265 (6).

The subjects were divided into two groups. The single viral infection group had only one virus reported via PCR, and the multiple viral infection group had two or more viruses reported via PCR.

This study was approved by the Clinical Research Ethics Committee of Ege University Faculty of Medicine (20-9.1T/48). The patients and/or parents of patients agreed to participate in this study after being informed of its purpose.

Respiratory virus examination by polymerase chain reaction test samples

Samples were taken from all children by a nasopharyngeal swab or an endotracheal aspirate in patients who were intubated within the first 24 hours of PICU admission. The samples were transported to the laboratory for PCR tests in a suitable container.

Viral nucleic acid isolation and amplification

Nucleic acids were isolated from the clinical samples with EZ1 Viruses Mini Kit v2.0 (Qiagen, Germany) protocol in EZ1 Advanced (Qiagen, Germany). The nucleic acids were stored at minus 80 degrees until amplification. Viral nucleic acid amplification was carried out with Fast Track Diagnostics (FTD) Respiratory Pathogen 21 tests FTD, Luxemburg) by means of multiplex real-time PCR. Influenza A, influenza B, influenza A, Rhinovirus, Coronavirus NL63, Coronavirus 229E, Coronavirus OC43, Coronavirus HKU1, Parainfluenza 1, Parainfluenza 2, Parainfluenza 3, Parainfluenza 4, hMPV A/B, Human bocavirus, Mycoplasma pneumoniae, RSV A/B (RSV), Adenovirus, Enterovirus and Human parechovirus can be determined with FTD Respiratory Pathogen 21 test. Amplifications of multiplex real-time PCR were determined with measurement of fluorescence radiation in Rotor-Gene (Qiagen, Germany). If the samples had a fluorescence signal, they were accepted as positive.

Statistical Analysis

The data were analyzed using the SPSS version 17.0 software. Conformity of variables to the normal distribution was examined with analytical methods such as the Kolmogorov-Smirnov/Shapiro-Wilk tests. Categorical data are presented as percentages, numerical data with Gaussian distribution are presented as mean ± standard deviation, and abnormally distributed data are presented as median (interquartile range). The Mann-Whitney U test was used to compare binary groups (single virus group vs. multiple virus group) in continuous data. Pearson's chi-square or Fisher's Exact test was used in the analysis of categorical data. Statistical results were considered significant for p-values <0.05.

Results

From March, 2018 to March, 2020, 490 children were admitted to the PICU with diagnoses of LRTI and 468 of these patients (468/490, 95.5%) who had a PCR test were enrolled into this study. Positive PCR tests were seen in 136 (29%, 136/468) of these patients. Of the 136 specimens included in this study, 91 (66.9%) were nasopharyngeal

swabs and 45 (33.1%) were endotracheal aspirates. As can be seen in Table I, Rhinovirus and RSV were the most commonly identified viruses (44.1% and 35.2%, respectively) in our cohort. Two viruses were detected in thirty-nine (28.6%) of the samples via PCR tests. Among the 39 children with viral co-infections, Rhinovirus was identified in 24 (61.5%) samples. Rhinovirus and RSV co-infection was the most common combination (10/39, 25.6%) in our cohort; followed by Rhinovirus and Enterovirus (6/39, 15.3%); Rhinovirus and Bocavirus (4/39, 10.2%); and RSV and Coronavirus (3/39, 7.6%). Bacterial co-infection was identified by endotracheal culture in 23 patients (16.9%). The most commonly detected organisms included *Streptococcus pneumoniae* (9 patients), *Staphylococcus aureus* (6 patients), *Haemophilus influenzae* (5 patients), *Moraxella catarrhalis* (2 patients), and *Pseudomonas aeruginosa* (1 patient). There was no statistically significant difference in the rate of confirmed bacterial infection between the single and multiple viral infection groups. The rate of empirical use of antibiotics was 76.2%.

The median age of the patients was 9 months [interquartile range (interquartile range IQR), 28.7 months]. One hundred and twenty-one patients (92%) were under 5 years of age and 77 patients (56.6%) were under 1 year of age. Seventy-nine of the 136 patients (58.1%) were male and 57 of the 136 children (41.9%) had an underlying medical condition, most commonly cardiovascular or neurologic diseases. The demographic and clinical characteristics of the patients are presented in Table II.

Fifty-seven children (42%) required intubation prior to PICU admission. Invasive ventilation was required in 57 children (41.9%) with a median duration of mechanical ventilation of 6 days (range, 1-41 days). Non-invasive ventilatory (NIV) support (either high-flow nasal cannula or bilevel positive airway pressure) was required in 23 children (16.9%). The median length of PICU stay was 5 days (range, 1-60 days). One patient (0.7%) required extracorporeal membrane oxygenation support. The median PRISM score was 9 (IQR 10), and 7 (5%) of the patients died during their PICU stay.

Table III shows the comparison between the single and multiple viral infection groups for clinical variables. There was no statistically significant difference in age, sex, chronic disease and mortality between the single and multiple viral infection groups. The multiple viral infection group had higher PRISM scores than the single virus infection group (10 vs. 7, respectively, p=0.009). In the multiple viral infection group, the invasive ventilatory support rate (56.4% vs. 36.1%, p=0.030) and the NIV support rate (43.5% vs. 6.1%, p=0.018) were significantly higher than in the single viral infection group.

Table I. Pathogens in single and multiple viral lower respiratory tract infections in pediatric intensive care unit

Microbiology result	Single infection (n)	Co-infection (n)	Total (n, %)
Rhinovirus	35	25	60 (44.1)
RSV	31	17	48 (35.2)
Bocavirus	5	9	14 (10.2)
Influenza A	8	5	13 (9.5)
Parainfluenza	6	5	11 (8)
Coronavirus	2	8	10 (7.3)
Enterovirus	0	6	6 (4.4)
Adenovirus	1	4	5 (3.6)
Influenza B	4	1	5 (3.6)
hMPV	4	0	4 (2.9)

RSV: Respiratory Syncytial Virus, hMPV: Human Metapneumovirus

Table II. Study population characteristics (n=136)

Characteristics	n (%) or median (interquartile range)
Gender, male	79 (58.1)
Age, months	9 (28.7)
<1 year	77 (56.6)
<5 years	121 (92)
Underlying disease	57 (41.9)
Cardiac	21 (15.4)
Neurologic	12 (8.8)
Respiratory	12 (8.8)
Immunocompromised	7 (5.1)
Prematurity	5 (3.6)
PRISM score	9 (10)
PICU length of stay, (days)	5 (8.7)
Hospital length of stay, (days)	7 (10.7)
Duration of invasive ventilation (days)	6 (7.5)
PARDS, n (%)	12 (8.8)
Bacterial co-infection, n (%)	23 (16.9)
Antibiotic treatment, n (%)	78 (57.3)
Antiviral treatment, n (%)	37 (27.2)
Mortality, n (%)	7 (5.1)

PRISM: Pediatric risk of mortality, PICU: Pediatric intensive care unit, PARDS: Pediatric acute respiratory distress syndrome

Table III. Comparison of clinical and disease severity variables between single and multiple viral infection group

	Single viral infection (n=97)	Multipl viral infection (n=39)	p-value
Gender, male. n (%)	52 (53.6)	27 (69.2)	0.095
Age, months, median (IQR)	9 (27.7)	6 (33)	0.334
Underlying disease, n (%)	42 (43.2)	15 (38.4)	0.605
Non-invasive ventilatory support, n (%)	6 (6.1)	17 (43.5)	**0.018**
Invasive ventilatory support, n (%)	35 (36.1)	22 (56.4)	**0.030**
Invasive ventilation days, median (IQR)	5 (7)	8 (9.25)	0.325
PICU length of stay, median day (IQR)	9 (12)	11 (14.75)	0.410
Hospital length of stay, median day (IQR)	12 (15)	14 (20.5)	0.524
PRISM score, median (IQR)	7 (10.5)	10 (12)	0.009
PARDS, n (%)	8 (8.2)	4 (10.3)	0.742*
Bacterial co-infection, n (%)	17 (17.5)	6 (15.4)	0.763
Mortality, n (%)	5 (5.2)	2 (5.1)	1.000*

*Fisher's exact test
IQR: Interquartile range, PICU: Pediatric intensive care unit, PARDS: Pediatric acute respiratory distress syndrome, PRISM: Pediatric risk of mortality

Discussion

In this study, we showed that multiple viral LRTIs were associated with higher rates of invasive and NIV support requirements and higher PRISM scores at admission. However, there was no statistically significant difference in PICU length of stay and mortality between the single and multiple viral infection groups. Although there are controversial reports about the association between multiple viral infections and disease severity, this may be related to heterogeneities in the patient populations and disease severity definitions (7-10). In PICU specific studies, no association has been reported between viral co-infection and clinical outcomes, including the need for mechanical ventilation and mortality (4,5).

Consistent with the previous studies, our study revealed that the prevalence of multiple viruses was 28.6% (4,7,11,12). Rhinovirus and RSV co-infection was the most common combination (25.6%) in our cohort, as has been reported previously (5,7). It is well known that RSV is one of the main agents associated with upper and LRTI in infants and it has been shown to cause more serious illness than other respiratory viruses (13). The association between specific co-infections and disease severity has been reported in previous studies. Semple et al. (8) reported that the hMPV and RSV co-infection is associated with severe bronchiolitis and it resulted in a 10-fold increase in the risk of PICU admission. Mansbach et al. (14) reported that those children with RSV/Rhinovirus co-infections had significantly longer lengths of hospital stay in comparison to children with RSV-only infections. On the other hand, in a study which compared the disease severity between single and multiple viruses, the authors reported that infants with RSV alone had longer lengths of hospital stay in comparison to those with RSV/Rhinovirus co-infection (15). It has been reported in the literature that milder Rhinovirus infections may have a protective effect (16).

Although there are conflicting results on the association between specific viral co-infections and disease severity, it is important to be aware that children with multiple viral infections may need invasive/non-invasive ventilatory support. Richard et al. (7) reported that infants with viral co-infection were 2.9 times more likely to be admitted to PICUs than those with single viral infections. In a previous study, they demonstrated that, in children with severe bronchiolitis, the use of early NIV resulted in an effective PCO_2 reduction and speculated that early NIV support prevents airway collapse and disease progression (17). In this patient population, early NIV support in the pediatric ward or pediatric emergency department may decrease PICU admissions. In another study which screened children <1 year of age who were admitted to a PICU, no differences were reported in comorbidities between single and multiple virus infections, which is consistent with our findings (18).

Epidemiological studies are generally conducted among symptomatic children by respiratory viral panels. In a large scale community study, the rates of asymptomatic respiratory virus infection were reported to be between 69% and 74% among different age categories (19). The rate of Rhinovirus colonization prevalence in the nasopharynx of asymptomatic children was between 5% and 18% (20,21). While respiratory virus panels allow us to identify treatable pathogens such as influenza, high rates of asymptomatic infection may lead to misjudgment.

In PICUs, approximately 50% of antibiotic use is inappropriate, and antibiotic overuse results in antibiotic resistance, increased costs, and drug toxicities (22).

Previous studies have demonstrated that a high percentage of children received empirical antibiotics (up to 100% in intubated children with bronchiolitis) (23,24). Respiratory virus examination with the PCR method not only provides epidemiological data, but also guides us on the use of oseltamivir. However, it does not affect the decision to use antibiotics. Although there are molecular methods which can be used for viral-bacterial infection differentiation and antimicrobial treatment selection, they have not been able to take their place in clinical practice due to their high cost and low accessibility (25).

Chauhan and Slamon (10) reported that multi-viral infections had a higher association of culture positive bacterial infection in children who required invasive ventilation and a higher rate of radiologic pneumonia. Our results are consistent with studies reporting no differences in confirmed bacterial infection rates in those case of dual infections (18). In our study, the presence of bacterial co-infection was evaluated only in intubated children, it may explain our report of lower bacterial infection rates.

Study Limitations

There were several limitations to this study, mainly attributed to its retrospective design. The presence of bacterial co-infection was evaluated only in intubated children, considering that the clinical definition of bacterial infection may lead to selection bias.

Conclusion

Lower respiratory multi-viral infections are associated with increased invasive and NIV support requirements. Close monitoring in a unit where support can be provided is essential for infants with multi-viral LRTIs. In the future, investigations which aim to identify the association between unfavorable outcomes and specific co-infections are needed.

Ethics

Ethics Committee Approval: This study was approved by the Clinical Research Ethics Committee of Ege University Faculty of Medicine (20-9.1T/48).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: P.Y.Ö., B.K., Design: P.Y.Ö., B.K., Data Collection or Processing: H.F.A., İ.E., Analysis or Interpretation: C.Ç., Writing: P.Y.Ö.

Conflict of Interest: The authors declared that there were no conflicts of interest.

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Original Article

Assessment of the Knowledge Levels and Attitudes of Physicians Regarding the Management of Acute Seizures in Pediatric Patients

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ABSTRACT

Aim: To evaluate the knowledge levels and attitudes of physicians regarding acute management of seizures in pediatric patients.

Materials and Methods: A self-administered questionnaire was distributed electronically to physicians. The participants were divided into 3 groups according to the estimated number of patients managed by the physician due to acute seizures per year; i) group 1: ≤10, ii) group 2: 11-50, iii) group 3: ≥51. Also, the participants were categorized whether they were pediatricians or not. Demographical features, and administration details regarding first- and second-line therapy were questioned. Rates of correct answers were compared between the groups.

Results: A total of 400 physicians responded to the questionnaire. Precisely, 74.5% of participants were pediatricians. The time point t_1 for tonic-clonic status epilepticus (SE) and focal SE with impaired consciousness were the least known details. Rates of correct answers to questions of the maximum number of benzodiazepines in case of ongoing seizures ($p<0.001$), intravenous diazepam dose ($p=0.017$), and diazepam infusion time ($p=0.034$) were significantly higher in group 3. Also, there was a tendency to administer lower doses of levetiracetam ($p=0.003$) and phenytoin ($p>0.001$), and prefer longer durations for phenytoin ($p=0.003$) in group 1 and group 2. Rates of correct answers to questions regarding the approach to patients who presented during the postictal period ($p<0.001$), the time point t_1 for tonic-clonic SE ($p=0.07$), the maximum number of benzodiazepines in case of ongoing seizures ($p<0.001$), diazepam infusion time ($p<0.001$), and co-administered liquid for phenytoin ($p=0.043$) were higher in pediatricians. Additionally, there was a significant tendency to administer lower doses of levetiracetam ($p<0.001$) and phenytoin ($p<0.001$), and prefer longer durations for levetiracetam ($p<0.001$) and phenytoin ($p<0.001$) in physicians other than pediatricians.

Conclusion: There is a wide variation in knowledge levels and attitudes among physicians. Post-graduation education programs focusing on the least-known and important details are needed.

Keywords: Seizure, acute management, children and adolescents, knowledge level, attitude

Introduction

A seizure is defined as "a transient occurrence of signs and symptoms owing to abnormally excessive or synchronous neuronal activity in the brain" (1). The estimated risk of experiencing any kind of seizure during the whole lifetime of an individual is approximately 8% (2). Its prognosis is associated with age, etiology, and duration of the seizure (3,4). International League Against Epilepsy proposed two operational dimensions in 2015 as follows: the time point t_1 (TP-t_1) indicates the time when pharmacological treatment should be initiated; the time point t_2 (TP-t_2) indicates the time when long-

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term consequences may appear. The TP-t_1s for tonic-clonic status epilepticus (SE), focal SE with impaired consciousness, and absence SE are 5 minutes, 10 minutes, and 10-15 minutes, respectively. The TP-t_2 for tonic-clonic SE is 30 minutes, and the time point t_2 for focal SE with impaired consciousness is 60 minutes. However, the TP-t_2 for absence SE is unknown (1). The first-line therapy consists of benzodiazepines. Intravenous administrations of levetiracetam, phenytoin, phenobarbital, and valproic acid are the options for the second-line therapy. Since the rapid termination of the seizure is crucial, physicians must have the adequate knowledge of the seizure management (5). However, there are not many studies investigating the knowledge level of the physicians about the acute seizures (6-9). To perform postgraduate programs, it is important to reveal their knowledge levels. Herein, we aimed to evaluate the knowledge level and attitudes of the physicians regarding the management of acute seizures in pediatric patients.

Materials and Methods

We have obtained the approval of the Aydın Adnan Menderes University Faculty of Medicine Clinical Research Ethics Committee (date: 20/01/2022; approval no: 2021/200) outlined in the Second Revision of WMA Declaration of Helsinki-Ethical Principles for Medical Research Involving Human Subjects. A self-administered questionnaire was written in Turkish with a cover letter, and reviewed by two independent pediatric neurologists and a pediatrician (Appendix 1). Afterwards, the questionnaires were distributed electronically to physician WhatsApp groups in Turkey. Informed consent was taken from the participants. We aimed to reach physicians who had a high possibility to treat children with acute seizures (practicing in speciality/subspecialities of pediatrics, general medicine, specialites of family medicine, neurology, neurosurgery, anesthesiology and reanimation). Physicians who agreed to respond to the items of the questionnaire were included in the survey. Data about demographic features including age, gender, and years of experience were collected. The participants were divided into three groups according to the estimated annual number of pediatric patients with acute seizures they treated as follows: i) group 1: ≤10, ii) group 2: 11-50, and iii) group 3: >50. Also, the participants were categorized whether they were pediatricians or not. The self-confidence of the physicians, administration details of the medications in the first-, and the second-line therapies were questioned. The correct answers to the questions regarding the durations of TP-t_1 for tonic-clonic SE and focal SE with impaired consciousness were accepted as 5 minutes, and 10 minutes, respectively. The correct answers to the questions relating to the "approach to the patients who presented during the postictal period", and "who started to seize in the hospital" were "investigating the etiology after initial stabilization steps", and "initial stabilization steps", respectively (1,5). The appropriate choice for the first-line therapy is benzodiazepines. In the first-line therapy, benzodiazepines can not be administered more than twice. The appropriate dose, maximum intravenous dose, and infusion rate for diazepam should be 0.15-0.2 mg/kg/dose, 10 mg/dose, and 5 mg/min, respectively. The appropriate dose, minimum infusion time, and suitable administration solution for phenytoin should be 20 mg/kg/dose, 20 minutes, and 0.9% sodium chloride, respectively (5,10,11). The appropriate loading IV infusion dose of levetiracetam is 40-60 mg/kg/dose and it should be administered within 15-20 minutes (5,12).

Statistical Analysis

Statistical analysis was performed using SPSS version 22 (IBM Corporation, Armonk, NY, USA). The sample size was calculated as three hundred eighty four by using stat calc (Epi Info) at p=0.5 d:0.05 and within 95% confidence interval. Descriptive variables were expressed as percentages (%), the means ± standard deviation, or medians with maximum and minimum values in parentheses. A chi-squared or Fisher's exact test was used for categorical variables, and the Mann-Whitney U test and Kruskal-Wallis H test were followed by a Dunn's post-hoc test for quantitative data after normality of distribution was assessed using the Kolmogorov-Smirnov test. A p-value <0.05 was considered to indicate statistical significance.

Results

A total of four hundred participants including 234 (58.5%) male, and 166 (41.5%) female physicians responded to the questionnaire. Precisely, 84.5% of the responders were pediatricians who remarked that they had self-confidence (Table I). Tables II, III and Figure 1 present the rates of answers to the questions. Details about the TP-t_1 for tonic-clonic SE (32.3%) and for focal SE with impaired consciousness (6.8%) were least known. The rates of correct answers to the questions of the maximum number of benzodiazepine administrations in case of ongoing seizures (p<0.001), intravenous

Table I. Demographical features of the physicians

	n/mean ± SD	%/median (min.-max.)
Gender		
• Female	166	41.5
• Male	234	58.5
Age	36.1±8.2	35.0 (24.0-67.0)
Experience in years	11.3±8.6	10.0 (0.1-42.0)
Speciality		
• Pediatrics	298	74.5
• Emergency medicine	20	5.0
• Family medicine	12	3.0
• General medicine	57	14.3
• Other	13	3.3
Having self-confidence regarding the management of acute seizures		
• Yes	338	84.5
• No	10	2.5
• Unsure	52	13.0
Estimated annual number of patients with acute seizures managed by the physician		
• ≤10	120	30.0
• 11-50	121	30.3
• ≥51	159	39.8

Descriptive variables are expressed as percentages (%), the means ± standard deviation, or medians with maximum and minimum values in parentheses
SD: Standard deviation, min.: Minimum, max.: Maximum

Table II. The rates of answers to the questions regarding the acute management of seizures

	n	%
Approach to the patient who presented during the postictal period after a 2-3 minutes lasting seizure		
• Investigating the etiology after initial stabilization steps (supports of the airway, breathing, and circulating)	**387**	**96.8**
• Intravenous administration of levetiracetam (loading and maintainence)	8	2.0
• Intravenous administration of phenytoin (loading and maintainence doses)	4	1.0
• Rectal diazepam	1	0.3
Approach to the patients who started to seize in the hospital (the first step should be chosen)		
• Rectal diazepam	11	2.8
• Initial stabilization steps (supports of the airway, breathing, and circulating)	**383**	**95.8**
• Blood glucose sampling	3	0.8
• Establishing an intravenous route	3	0.8
The time point t_1 for tonic-clonic SE (initiation of pharmacological threapy)		
• As soon as possible	80	20
• Within 2-3 min following the initial stabilization steps	189	47.3
• At 5. min following the initial stabilization steps	**129**	**32.3**
• At 10. min following the initial stabilization steps	2	0.5
The time point t_1 for focal SE with impaired consciousness (initiation of pharmacological threapy)		
• As soon as possible	176	44.0
• Within 2-3 min following the initial stabilization steps	110	27.5
• At 5. min following the initial stabilization steps	87	21.8
• At 10. min following the initial stabilization steps	**27**	**6.8**
The appropriate medication in the first-line therapy		
• Benzodiazepin (intravenous/buccal/intranasal/rectal)	**346**	**86.5**
• Intravenous administration of levetiracetam (loading and maintainence doses)	37	9.3
• Intravenous administration of phenytoin (loading and maintainence doses)	16	4.0
• Phenobarbital (by nasogastric tube)	1	0.3
The maximum number of benzodiazepine administrations that can used in case of ongoing seizure in the first-line therapy		
• One	16	4.0
• Two	**223**	**55.8**
• Three	161	40.3

The rate of correct answers are written in bold characters

diazepam dose (p=0.017), and diazepam infusion time (p=0.034) were significantly higher in group 3 than in the other groups. Also, there was a significant tendency to administer lower doses of levetiracetam (p=0.003), and phenytoin (p>0.001), and to infuse IV phenytoin for longer periods of time (p=0.003) in group 3 than in the other groups (Table IV). The rates of correct answers to the questions regarding the approach to the patients who presented during the postictal period (p<0.001), the TP-t_1 for tonic-clonic SE (p=0.07), the maximum number of benzodiazepine administrations in case of ongoing seizures (p<0.001), infusion times for diazepam (p<0.001), and levetiracetam (p<0.001), suitable administration solution for phenytoin (p=0.043) were significantly higher among pediatricians than non-pediatricians (Table V). Additionally, there was a significant tendency to administer lower doses of levetiracetam (p<0.001), and phenytoin (p<0.001), and administer phenytoin with a longer infusion time at a dose of 20 mg/kg (p<0.001) among physicians other than pediatricians.

Table III. The rates of answers to the details regarding the knowledge of pharmacological therapy

	n	%
Intravenous doses of diazepam (per kilogram)		
• 0.15-0.2 mg/kg/dose	**322**	**80.5**
• 0.5 mg/kg/dose	69	17.3
• 1 mg/kg/dose	9	2.3
Maximum dose of intravenous diazepam (adult dose)		
• 5 mg/dose	158	39.5
• 10 mg/dose	**213**	**53.3**
• 20 mg/dose	29	7.3
Intravenous infusion rate of diazepam		
• Rapid enjection (bolus)	142	35.5
• 5 mg/min infusion	**214**	**53.5**
• 30 min infusion	44	11.0
Intravenous levetiracetam loading dose		
• 10 mg/kg/dose	60	15.0
• 20 mg/kg/dose	185	46.3
• 40-60 mg/kg/dose	**155**	**38.8**
Levetiracetam infusion time		
• Rapid enjection (bolus)	51	12.8
• 15-20 min infusion	**202**	**50.5**
• 30 min infusion	147	36.8
Intravenous phenytoin dose		
• 10-15 mg/kg/dose	316	79.0
• 20 mg/kg/dose	**80**	**20.0**
• 40 mg/kg/dose	4	1.0
Minimum infusion time for phenytoin dose of 20 mg/kg		
• 10 min	50	12.5
• 20 min	**159**	**39.8**
• 30 min	191	47.8
Suitable diluent administration solution for intravenous phenytoin		
• Dextrose + ringer lactate	6	1.5
• 0.9% sodium chloride	**325**	**81.3**
• 5% dextrose in water	66	16.5
• 10% dextrose in water	3	0.8

The rate of correct answers are written in bold characters.

Table IV. Comparison of rates of correct answers to the questions regarding the management of acute seizures according to the estimated annual number of patients with acute seizures managed by physicians

	Group 1 ≤10 (n=120) n (%)	**Group 2 11-50 (n=121) n (%)**	**Group 3 ≥51 (n=159) n (%)**	**p-value**
Approach to the patient who presented during the postictal period	115 (95.8)	115 (95.8)	157 (98.7)	0.178
Approach to the patient whose seizure initiated in the hospital	113 (94.2)	115 (95.0)	155 (97.5)	0.356
The time point t_1 for tonic clonic seizures	29 (24.2)	40 (33.1)	60 (37.7)	0.055
The time point t_1 for focal seizures with impaired consciousness	7 (5.8)	5 (4.1)	15 (9.4)	0.192
The appropriate medications in the first-line therapy	104 (86.7)	102 (84.3)	140 (88.1)	0.659
The maximum number of benzodiazepines in case of ongoing seizures	55 (45.8)	60 (49.6)	108 (67.9)	**<0.0001**
Knowledge level regarding intravenous diazepam administration • Appropriate intravenous dose • Maximum dose • Infusion time	 87 (72.5) 55 (45.8) 53(44.2)	 98 (81.0) 64 (52.9) 66 (54.5)	 137 (86.2) 94 (59.1) 95 (59.7)	 **0.017** 0.088 **0.034**
Knowledge level regarding levetiracetam administration • Appropriate intravenous dose • Infusion time	 35 (29.2) 56 (46.7)	 43 (35.5) 56 (46.3)	 77 (48.4) 90 (56.6)	 **0.003** 0.140
Knowledge level regarding pheniytoin administration • Appropriate intravenous dose • Suitable administration solution • Minimum Infusion time	 57 (47.5) 92 (76.7) 37 (30.8)	 69 (57.0) 98 (81.0) 43 (35.5)	 116 (73.0) 135 (84.9) 79 (49.7)	 **<0.0001** 0.217 **0.003**

Kruskal-Wallis H test were followed by a Dunn's post-hoc test for quantitative data following an assessment of normality in the Kolmogorov-Smirnov test

Table V. Comparison of rates of correct answers to the questions regarding the management of acute seizures according to the speciality

	Pediatricians (n=298) n (%)	**Physicians other than pediatricians (n=102) n (%)**	**p-value**
Approach to the patient who presented during the postictal period	295 (99.0)	92 (90.2)	**<0.001**
Approach to the patient whose seizure initiated in the hospital	288 (96.6)	95 (93.1)	0.130
The time point t_1 for tonic-clonic SE	107 (35.9)	22 (21.6)	**0.007**
The time point t_1 for focal SE with impaired consciousness	18 (6.0)	9 (8.8)	0.334
The appropriate medication in the first-line therapy	258 (86.6)	88 (86.3)	0.938
The maximum number of benzodiazepines in case of ongoing seizures in the first-line therapy	182 (61.1)	41 (40.2)	**<0.001**
Knowledge level regarding intravenous diazepam administration • Appropriate intravenous dose • Maximum dose • Infusion time	 245 (82.2) 166 (55.7) 166 (59.1)	 77 (75.5) 47 (46.1) 38 (37.3)	 0.139 0.093 **<0.001**
Knowledge level regarding levetiracetam administration • Appropriate intravenous dose • Infusion time	 131 (44) 164 (55.4)	 24 (23.5) 37 (36.3)	 **<0.001** **<0.001**
Knowledge level regarding phenytoin administration • Appropriate intravenous dose • Suitable administration solution • Infusion time	 209 (70.1) 249 (83.6) 138 (46.3)	 33 (32.4) 76 (74.5) 21 (20.6)	 **<0.001** **0.043** **<0.001**

Mann-Whitney U test for quantitative data following an assessment of normality in the Kolmogorov-Smirnov test

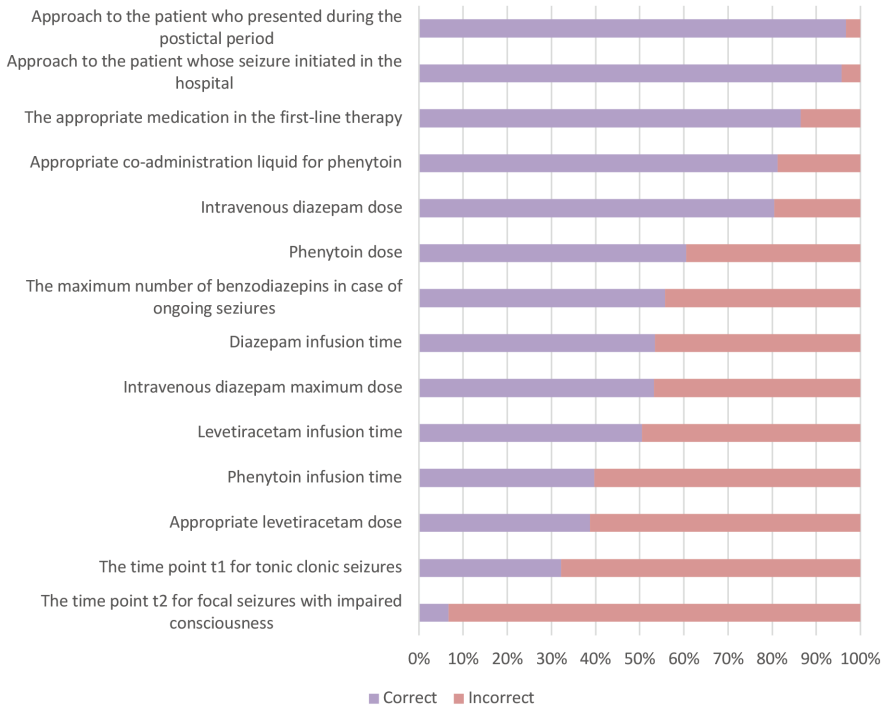

Figure 1. Rates of correct and incorrect answers to the questions regarding the management of acute seizures in pediatric patients

Discussion

The major findings in the current study were as follows; i) the details of the TP-t_1 for tonic-clonic SE (32.3%) and focal SE with impaired consciousness (6.8%) were the least known, ii) 40.5% of the participants wrongly stated that benzodiazepine should be administered at most three doses in case of ongoing seizures, iii) there was a tendency to administer lower doses of levetiracetam, and phenytoin, and use longer infusion time for phenytoin, iv) there was a wide distribution in knowledge levels and attitudes between the groups and between pediatricians and non-pediatricians.

The current study is the first study that evaluated the knowledge level of physicians regarding the time points. We demonstrated that details regarding Tp-t_1 for tonic-clonic SE (32.3%) and focal SE with impaired consciousness (6.8%) were the least known, and there were no significant differences between the groups in terms of knowledge levels of these questions. The rate of correct answers to the question of TP-t_1 for tonic-clonic SE was significantly higher among pediatricians. Most of the incorrect answers to both questions were related to the earlier initiation of pharmacological treatment. Although earlier onset of pharmacological therapy may not lead to poor outcomes compared to delay in treatment, it may increase adverse effects such as respiratory depression (13-18). After examining the efficacy of initial pharmacological treatment in 26 randomized controlled trials, intravenous administrations of lorazepam and diazepam were proposed as the efficacious options (level A evidence). Also, non-intravenous benzodiazepines (rectal diazepam, intramuscular midazolam, and buccal midazolam) were suggested as probably effective medications (level B evidence) (5). Experimental models have proposed that inhibitory $GABA_A$ receptors that are located on the postsynaptic membrane move into clathrin-coated vesicles, and N-methyl-D-aspartate (NMDA) receptors are mobilized into the membrane in case of seizure. Therefore, it has been proposed that benzodiazepines are effective in the early minutes (within 5-20 minutes) of SE (19,20).

Intravenous lorazepam and diazepam can be administrated twice, in case of ongoing seizures (5). In the current study, 86.5% of the participants responded correctly to the question inquiring the appropriate medication to be administered in the first-line therapy. There were no significant differences between the three groups, and also between pediatricians and non-pediatricians in terms of correct response rates concerning this question. Since the inadequate knowledge level of physicians regarding this emergency situation may lead to failure of seizure

control, the rate of correct answers should be raised to maximum. The question regarding the maximum number of benzodiazepine administrations in case of ongoing seizures was responded correctly by 55.8% of the participants and 40.2% of the participants responded incorrectly as "three times". However, the effects of benzodiazepines may diminish in the later stages of SE, and an overdose of benzodiazepines may lead to respiratory depression (21).

In the current study, 80.5% of the participants responded correctly to the question related to the appropriate dose for intravenous diazepam. The rate of correct answers was higher in group 3. Most of the incorrect answers were related to higher doses that may lead to respiratory depression. Non-intravenous benzodiazepines are also effective and it is suggested especially if an intravenous line is not available (level B evidence) (5). Rectal diazepam administration (0.5 mg/kg/dose) may be easier to remember. Rectal tubes containing 5 mg, and 10 mg diazepam are appropriate for an infant weighing <10 kg, and a child weighing ≥10 kg, respectively (5). Ease of remembering may lead to a preference for rectal diazepam, especially in physicians who managed pediatric patients with acute seizures more infrequently. Precisely, 53.2% of the participants responded correctly to the question of appropriate maximum dose of diazepam and most of the incorrect answers were stated as 5 mg/dose (39.5%). There were no significant differences between the three groups, and between pediatricians and non-pediatricians in terms of response rates related to this question. However, the administration of lower doses of diazepam may fail to control seizures (5,22). In the current study, 53.5% of the participants responded correctly to the question related to appropriate diazepam infusion time. The rate of correct answers was significantly higher in group 3 and among pediatricians. Precisely, 35.5% of the participants have chosen the "rapid injection (bolus)" option which may lead to respiratory depression (23-25).

The second-line therapy should be initiated when the seizure persists up to 20 minutes. Intravenous fosphenytoin/phenytoin (level U evidence), valproic acid (level B evidence), and intravenous levetiracetam (level U evidence) are the recommended options (5). There have been studies comparing the effectiveness of phenytoin/fosphenytoin and levetiracetam in the second-line therapy. According to the results of the "Emergency treatment with Levetiracetam or Phenytoin in convulsive SE in children" trial, levetiracetam (40 mg/kg) was not significantly superior to phenytoin (20 mg/kg) in terms of cessation rate of convulsive seizures, the time taken to terminate convulsive seizures or adverse effects (26). Also, in Convulsive SE Paediatric Trial, there were no significant differences between levetiracetam (40 mg/kg, over 5 min) and phenytoin (20 mg/kg), in terms of intubation rates, length of intensive care unit, and hospital stay, and termination of the seizure (27). In the study of the Established SE Treatment Trial, the efficacy and safety of levetiracetam (60 mg/kg), fosphenytoin (20 mg/kg), and valproic acid (40 mg/kg) were compared. According to the results of this trial, any of the three drugs had no superiority over each other in the second-line therapy (17). However, due to some serious adverse effects such as acute hepatotoxicity or acute hepatic failure after administration of valproic acid may occur, utilization of valproic acid is limited especially in children <2 years old, and in the presence of a higher risk of inborn error of metabolism (28,29). Thus, we questioned the administration details of phenytoin and levetiracetam in pediatric patients. In the current study, 38.8% of the participants responded correctly to the question of appropriate levetiracetam dose. Most of the incorrect answers consist of lower doses. The rate of correct answers was significantly higher in group 3 and among pediatricians. However, since higher doses of levetiracetam (40-60 mg/kg) were proposed in the previous trials, administration of lower doses may lead to failure of the treatment (17,26,27). Precisely, 50.5% of the participants answered correctly to the question of levetiracetam infusion time and the rate of the correct answers was significantly higher among pediatricians. Since 36.8% of the participants have preferred a longer duration of infusion (30 min), some patients may not benefit from the advantage of rapid achievement of high serum levels. The rates of correct answers to the questions of "maximum loading dose per kilogram" and "infusion time of fosphenytoin/phenytoin" were 20.0% and 39.8%, respectively. Additionally, the rates of correct answers to both questions were significantly higher in group 3 and also among pediatricians. Among all participants, there was a significant tendency to administer lower doses of phenytoin (10-15 mg/kg) and prefer longer durations of infusion (30 min). Although these tendencies do not increase the risk of adverse effects, the possibility of rapidly terminating seizure may decrease. Most (81.3%) of the participants responded correctly to the question of suitable administration solution and the relevant knowledge level was significantly higher among pediatricians. Since phenytoin becomes unstable with

liquids containing dextrose (11), incorrect administration of phenytoin may lead to the poor seizure control.

There are few studies evaluating the knowledge level and attitudes of physicians regarding asthma, SE, and febrile seizures. Mikhaeil-Demo et al. (9) evaluated the improvement of the knowledge level of neurology residents regarding SE after using a stimulation-based mastery learning curriculum. According to the results, after the intervention, significant improvements were observed in evaluating the relevant medical history, stabilizing patients, ordering first- and second-line treatments correctly, evaluating the necessity of neuroimaging, and re-evaluating the case (9). Yilmaz et al. (30) suggested that recommending a prophylactic treatment for febrile seizures (intermittent/long-term) differed even within the same speciality. Similarly, Bashiri et al. (31) demonstrated that there was a wide variation in knowledge levels and attitudes regarding febrile seizures in different specialities. Additionally, they proposed that a significant number of physicians should receive further education on this issue (31). Also, it was proposed that education is necessary concerning the management of asthma (32). Similarly, the results of the current study indicate the necessity for postgraduate education programs regarding the acute management of seizures.

Study Limitations

This is the first study that questioned the knowledge level of physicians regarding the acute management of seizures in children. However, our study had some limitations. First, the majority of the responders were pediatricians that may erroneously lead to yielding results indicating a higher knowledge level. Second, we were only able to provide estimates, as information on the annual number of pediatric patients managed by physicians was obtained based on their own reports. Third, the level of knowledge regarding the administration of benzodiazepines other than midazolam was not obtained. This may result in the study not fully reflect the level of knowledge for all benzodiazepines (e.g., diazepam). In addition, since the number of studies evaluating the level of knowledge on this subject is limited, we compared studies examining the knowledge levels of physicians on different subjects. These studies may not be fully comparable with our study in some aspects.

Conclusion

In conclusion, there is a wide variation in knowledge levels and attitudes among physicians. Organizing education programs focusing on the least known and/or important details for physicians is necessary for the acute management of seizures in pediatric patients.

Ethics

Ethics Committee Approval: We have obtained the approval of the Aydın Adnan Menderes University Faculty of Medicine Clinical Research Ethics Committee (date: 20/01/2022; approval no: 2021/200).

Informed Consent: Informed consent was taken from the participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Design: M.A., S.Ö., A.T., Data Collection and/or Processing: M.A., Analysis and/or Interpretation: S.Ö., Literature Search: A.T., Writing: M.A.

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Appendix 1: English version of the questionnaire
Assessment of the knowledge level and attitudes of physicians regarding the management of acute seizures in pediatric patients
Dear participants;
We aimed to evaluate the knowledge level of physicians regarding the acute management of seizures in pediatric patients. The targeted speciality groups consist of pediatrics, emergency medicine, family medicine, general medicine, neurology, neurosurgery, anesthesia and reanimation. According to the study results, postgraduation education programs are going to be organized. Your answers to the questionnaire will be anonymous and will not be known to us. Answering the questionnaire takes ten minutes. We appreciate your participation.
1. If you want to participate in this study, please click yes, and continue.*
• Yes
• No
2. Age* .....
3. Gender *
• Female
• Male
4. How many years have you been working as a physician? .....
5. In which speciality do you work or study?
• General medicine
• Pediatrics
• Emergency medicine
• Anesthesia and reanimation
• Neurology
• Neurosurgery
• Familiy medicine
6. Approximately, how many children presenting with acute seizures do you treat per year? .....
7. Do you have the self-confidence regarding the management of acute seizures?
• Yes
• No
• Unsure
8. How would be your approach to the patient who presented during the postictal period after a 2-3 minutes lasting seizure?
• Investigating the etiology after initial stabilization steps (supports of the airway, breathing, and circulating)
• Intravenous administration of levetiracetam (loading and maintainence)
• Intravenous administration of phenytoin (loading and maintainence doses)
• Rectal diazepam
9. How would your approach to the patients who started to seize in the hospital (The first step should be chosen)
• Rectal diazepam
• Initial stabilization steps (supports of the airway, breathing, and circulating)
• Blood glucose sampling
• Establishing an intravenous route
10. When would you initiate pharmacological therapy in a seizing child (tonic-clonic)? (The time point t1 for tonic-clonic SE)
• As soon as possible
• Within 2-3 min following the initial stabilization steps
• At 5. min following the initial stabilization steps
• At 10. min following the initial stabilization steps
11. When would you initiate pharmacological therapy in a seizing child (focal SE with impaired consciousness)? (The time point t1 for focal SE with impaired consciousness)
• As soon as possible
• Within 2-3 min following the initial stabilization steps
• At 5. min following the initial stabilization steps
• At 10. min following the initial stabilization steps

Appendix 1: Continued
12. Which anticonvusant would you chose in the first-line therapy?
• Benzodiazepin (intravenous/buccal/intranasal/rectal)
• Intravenous administration of levetiracetam (loading and maintainence doses)
• Intravenous administration of phenytoin (loading and maintainence doses)
• Phenobarbital (by nasogastric tube)
13. What is the appropriate dose for intravenous diazepam?
• 0.15-0.2 mg/kg/dose
• 0.5 mg/kg/dose
• 1 mg/kg/dose
What is the maximum dose (adult dose) of intravenous diazepam?
• Maximum 5 mg/dose
• Maximum 10 mg/dose
• Maximum 20 mg/dose
What is the appropriate infusion time for intravenous diazepam?
• Rapid enjection (bolus)
• 5 mg/min
• 30 dk iv infusion
14. How many doses of benzodiazepines would you administer in the first-line therapy in case of ongoing seizure?
• One
• Two
• Three
What is the appropriate intravenous phenytoin, in case of ongoing seizure?
• 10-15 mg/kg/dose
• 20 mg/kg/dose
• 40 mg/kg/dose
What is the minimum infusion time for a dose of 20 mg/kg phenytoin?
• Minimum 10 minutes
•· Minimum 20 minutes
• Minimum 30 minutes
What is the suitable diluent solution for intravenous phenytoin?
• Dextrose + ringer lactate
• 0.9% sodium chloride
• 5% dextrose in water
• 10% dextrose in water
15. What dose do you administer intravenous levetiracetam, in case of ongoing seizure?
• 10 mg/kg/dose
• 20 mg/kg/dose
• 40-60 mg/kg/dose
What is the appropriate infusion time for intravenous levetiracetam
• Rapid enjection (bolus)
• 15-20 min infusion
• 30 min infusion

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Original Article

Less Invasive Surfactant Administration Versus Intubation for Surfactant Delivery in Very Low Birth Weight Infants

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ABSTRACT

Aim: Routes for surfactant administration for respiratory distress syndrome (RDS) has changed from bolus endotracheal administration together with ongoing mechanical ventilation, to intubation-surfactant administration and rapid extubation (INSURE) method and finally to less invasive surfactant administration (LISA). In this study our aim was to compare respiratory outcomes of LISA and INSURE methods for surfactant delivery in very low birth weight (VLBW) infants.

Materials and Methods: This retrospective, single-center study was performed in a one year period in between March 2014-2015. Data of VLBW infants who had diagnosis of RDS and received surfactant treatment via LISA or INSURE techniques were analyzed. Primary outcome of the study was failure of non-invasive respiratory support. Secondary outcomes were bronchopulmonary dysplasia diagnosis and its severity, duration of mechanical ventilation via endotracheal tube, total number of surfactant administered, duration of hospitalization and duration of all sorts of non-invasive respiratory support. Non-invasive ventilatory support failure incidences of LISA group according to gestational ages were also analyzed.

Results: Fifty-nine VLBW infants in LISA group and 55 VLBW infants in INSURE group were analyzed. Need for intubation/reintubation (non-invasive ventilatory support failure) was significantly lower in LISA group (31.6% vs 49%, p=0.043). Duration of intubation was significantly longer in INSURE group [0 vs 4 days (median), p=0.001]. Both LISA and INSURE treated infants had similar moderate to severe BPD ratios (26.6% vs 32.7%, p=0.306). We did not observe any reported complications during application of both methods. Intubation ratios were lowest in the group with gestational ages 28-29 weeks (25%).

Conclusion: LISA technique for surfactant delivery to preterms with RDS is a safe method ending with lower rates of need for intubation/reintubation. Even if no difference in BPD incidences in between the two groups was observed at the 36th corrected gestational week, intubation duration of infants was significantly lower in LISA group.

Keywords: LISA, INSURE, surfactant, preterm infant

Introduction

Respiratory distress syndrome (RDS) is a common morbidity experienced in premature infants, having the major etiology of surfactant deficiency (1). This deficiency was described nearly 60 years ago and treatment of preterm infants with exogenous surfactant preparations has been one of the most important milestones in neonatology (2). Surfactant treatment, as the most effective evidence-based

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therapy for RDS, has been shown to reduce the risk of death and bronchopulmonary dysplasia (BPD) in preterm infants (3,4). Since its first use, routes for surfactant administration have changed from bolus endotracheal administration together with ongoing mechanical ventilation, to the intubation-surfactant administration and rapid extubation (INSURE) method and finally to less invasive surfactant administration (LISA) which enables infants to go on spontaneous breathing whilst having non-invasive respiratory support without facing the consequences of intubation (1,5). Even if infants are extubated shortly after receiving surfactant by the INSURE method, there is still a brief time of positive pressure ventilation (1). However, ventilator-induced lung injury poses many risks for the vulnerable lungs of preterm infants (6-10). Non-invasive ventilation is better compared to mechanical ventilation via endotracheal tube in terms of causing less alveolar injury (11-13).

A variety of guidelines in Europe indicate LISA to be the method of choice for surfactant administration (14-16). Additionally, there is significant interest in LISA worldwide with an increasing number of studies (17-22). Furthermore, LISA is a holistic non-invasive approach which aims to support the maximum capacity of the preterm to fulfill its potential during the adaptation period to extrauterine life (1). With the results of several randomized controlled trials, the need for mechanical ventilation was shown to decrease as a result of LISA (23-25). Not only intubation rates, but also the rate of BPD in LISA-treated preterm infants is low compared to international standards (1).

With all this knowledge, the aim of our study was to compare the respiratory outcomes of the LISA and INSURE methods for surfactant delivery in VLBW infants in a single center during a 1-year period.

Materials and Methods

This retrospective, single-center study was performed in the Izmir Medical Park Hospital Neonatal Intensive Care Unit (Izmir, Turkey). The medical records of preterm inborn or outborn infants who had been hospitalized in a one year period between March, 2014 and March, 2015 were reviewed for eligibility in this study. Data of VLBW infants who had received a diagnosis of RDS and received surfactant treatment via the LISA or INSURE techniques were analyzed. A flowchart of the included and excluded infants is shown in Figure 1. The RDS diagnosis and surfactant indications were made according to the guidelines of Turkish Neonatal Society (TNS) (26). Preterm infants who exhibited symptoms such as tachypnea, grunting, need for oxygen supplementation, and/or retractions were diagnosed as RDS. This diagnosis was confirmed by typical X-ray and blood gas findings. Surfactant was administered if the patient required ≥0.40 FiO_2 to maintain the target oxygen saturation level of 90-95% along with these signs and symptoms.

According to the individual guidelines of the unit, the decision for which technique to use was given by the attending physician. As an inclusion criterion, only those infants who received Poractant Alfa with a dose of 200 mg/kg and who had reached the 36th postmenstrual age were accepted. Infants with major congenital anomaly, who had received another type of surfactant preparation, who could not be extubated shortly after surfactant administration and/or whose digital medical records could not be obtained were excluded. This study was approved by Institutional Ethical Committee conducted in Buca Seyfi Demirsoy Training and Research Hospital (approval no: 2021/4-39 dated on 28.04.2021).

Both inborn and outborn infants were supported by delivery room teams who were experienced regarding pregnancy, and which risks needed to be identified before each delivery. Each unit had a checklist of materials which were required in the premature infants' delivery room for stabilization and/or resuscitation and the members of each team were competent in performing the recommended neonatal resuscitation program. Preterm infants with findings of respiratory insufficiency received non-invasive ventilatory support by NCPAP with at least 5 cm-H_2O through binasal prongs in the delivery room and during the transportation in cases where no urgent intubation indication emerged. Hypothermia was prevented and all of the preterm infants were monitored both clinically and by pulse-oximeters. All infants received prophylactic caffeine treatment according to the institutional guidelines and both the LISA and INSURE techniques were performed by the same team, similar to the methods described in the study conducted by Kanmaz et al. (21). In this technique, a 5F sterile and flexible nasogastric tube is used. The tube is shortened at 33 cm depth from the catheter hub. For the insertion depths, the gestational age of the infant is determinative. When the catheter is inserted through the vocal cords, 1.0, 1.5 and 2.0 cm insertions are performed for infants of 25-16, 27-28 and 29-32 gestational weeks, respectively. Standard laryngoscope and Miller 00 blade are used for direct laryngoscopy and catheter placement. The surfactant is drawn into a 5 mL syringe before direct laryngoscopy is performed. At this step, a

standard laryngoscope with a straight blade is used and the catheter is immediately removed as the planned amount of surfactant and 1 mL of air is applied. All throughout this procedure, the infants is kept on non-invasive ventilation support. As a standard policy, none of the infants receive premedication. The Jobe and Bancalari classification is used for BPD diagnosis and classification (22).

The primary aim of this study was to investigate the failure of non-invasive respiratory support. The secondary outcomes were BPD diagnosis and its severity, the duration of mechanical ventilation via endotracheal tube, the total amounts of surfactant administered, the duration of hospitalization and the duration of all sorts of non-invasive respiratory support. The non-invasive ventilatory support failure incidences of the LISA group according to gestational ages were also analyzed.

Statistical Analysis

Statistical analysis was conducted using the SPSS software for Windows version 25.0 (IBM, Armonk, NY: IBM Corp.) Descriptive statistics were used including mean (with standard deviations) and median [minimum-maximum (min.-max.)] for continuous variables, and counts (proportions) for categorical variables. The conformity of the data to the normal distribution was evaluated with the Kolmogorov-Smirnov test. Student's t-test and the Mann-Whitney U test compared continuous variables for parametric and non-parametric variables, respectively. The chi-square test was used for categorical variables. Statistical significance was considered if the p-value was <0.05.

Results

A total of 383 preterm infants were hospitalized during the period of this study and 189 of them were very low birth weight (VLBW) infants. The data of the VLBW infants who had RDS diagnoses and who received surfactant treatment via the LISA or INSURE methods were analyzed. Of the 130 infants who were treated via these two methods, 16 had insufficient medical records and finally, 59 VLBW infants in the LISA group and 55 VLBW infants in the INSURE group were analyzed (24-32 weeks) (Figure 1). The demographic characteristics and antenatal steroid rates of the infants in the two groups were similar (Table I).

The need for intubation/reintubation (non-invasive ventilatory support failure) was significantly lower in the LISA group (31.6% vs 49%, p=0.043). The total amount of surfactant administered was similar between the two groups (p=0.492). The duration of intubation was significantly longer in the INSURE group [0 vs 4 days (median), p=0.001]. The median duration of non-invasive ventilation was 11 (0-180) days for the LISA group and 20 (0-76) days for the INSURE group but this did not reach statistical significance (p=0.035). Both LISA and INSURE treated infants had similar moderate to severe BPD ratios (26.6% vs 32.7%, p=0.306) (Table II). The median duration of total oxygen support was similar in both groups at 37 (2-250) days for the LISA group and 48 (0-219) days for the INSURE group (p=0.039). However, there was a statistically significant difference regarding the duration of hospitalization between the two groups, being longer in the INSURE group (62.4±28.9 vs 87.5±46.4, p=0.001). We did not observe any reported complications during the application of either method. None of the infants experienced adverse events such as air leak, significant surfactant reflux, unilateral administration of surfactant or deterioration in vital signs leading to an interruption of the application.

When we performed subgroup analysis, classifying the LISA group according to their gestational ages, the intubation ratios were similar between the 3 subgroups (Table III).

Table I. Basic demographic characteristics of the infants

	LISA n=59	**INSURE n=55**	**p-value**
Gestational age, (weeks, mean±SD)	28.14±1.95	27.5±2.07	0.140
Birth weight (gr, mean±SD)	1106±292	1009±291	0.089
Maternal age, (years, mean±SD)	29.8±5.1	29.4±8.3	0.784
Antenatal steroid, n (%)	35 (59.3)	32 (58.2)	0.541
Gender, female, n (%)	27 (45.8)	27 (49)	0.739
Caesarean delivery, n (%)	50 (84.7)	45 (83.3)	0.842

LISA: Less invasive surfactant administration, INSURE: Intubate-Surfactant-Extubate

Table II. Incidence of short and long term respiratory morbidities			
	LISA n=59	**INSURE n=55**	**p-value**
Need for intubation/reintubation, n (%)	19 (31.6)	27 (49)	**0.043**
Total number of surfactant administration (mean±SD)	1.7±1.5	2.02±2.3	0.492
Duration of intubation (days, mean±SD)	3.68±11.3	15.7±23.7	**0.001**
Duration of noninvasive ventilation [days, median (min-max)]	11 (0-180)	20 (0-76)	0.035
Total duration of oxygen support [days, median (min-max)]	37 (2-250)	48 (0-219)	0.039
BPD (moderate, severe), n (%)	16 (26.6)	18 (32.7)	0.306
Duration of hospitalization (days, mean±SD)	62.4±28.9	87.5±46.4	**0.001**
LISA: Less invasive surfactant administration, INSURE: Intubate-Surfactant-Extubate, BPD: Bronchopulmonary dysplasia			

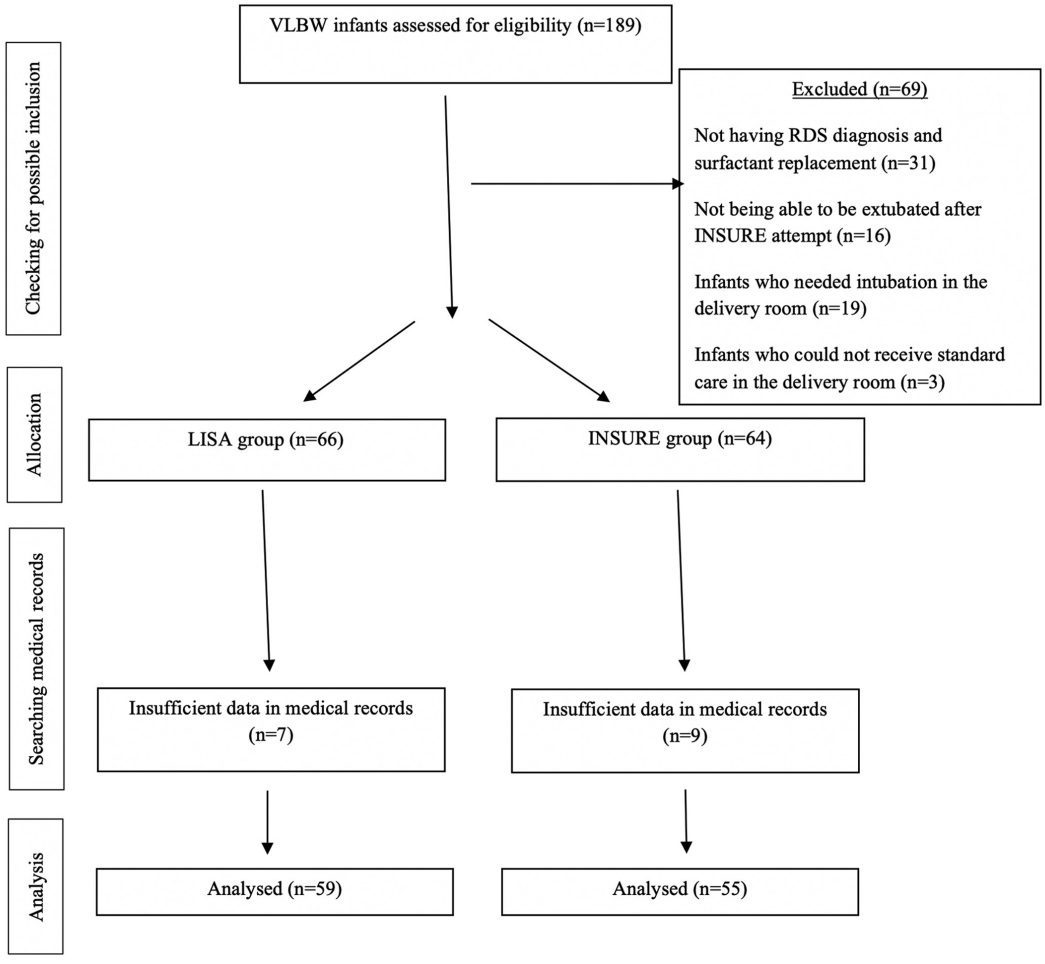

Figure 1. Flow diagram of included and excluded infants

Table III. Non-invasive ventilatory support failure incidences of LISA group according to gestational ages			
	Total number of infants, n (%)	**Intubation ratio (%)**	**p-value**
<26 wk GA	4 (6.8)	50	0.617
26-27^{6} wk GA	19 (32.2)	42.4	
28-29^{6} wk GA	20 (33.9)	25	
30-32 wk GA	16 (27.1)	31	
GA: Gestational age, wk: Week			

Discussion

In our study, we found lower intubation/reintubation rates with the LISA method compared to INSURE. In a meta-analysis comparing LISA with the standard method of surfactant delivery, data of 6 randomized controlled trials on 895 infants were evaluated and the LISA method was found to result in less need for mechanical ventilation, similar to the results of our study (27). In the same meta-analysis, BPD was evaluated together with death or the need for mechanical ventilation within 3 days of birth and it was seen that there was a reduction in these parameters with the use of LISA. In our study, moderate to severe BPD rates were less in the LISA group compared to the INSURE group. Our result is consistent with the findings of previous studies showing a reduction in BPD rates at the 36th week (21,28,29). As we did not include infants who died before the 36th gestational week and evaluated BPD rates among survivors, a composite outcome analysis was not possible in our study.

According to results of our study, the duration of mechanical ventilation was significantly lower in the LISA group. This finding was also consistent with the results of the studies conducted by Kanmaz et al. (21) and Göpel et al. (24) while several other studies reported similar durations of mechanical ventilation when the two groups are compared (15).

In our study, we preferred surfactant preparations of porcine origin with a starting dose of 200 mg/kg according to the recommendations of TNS and as a rescue treatment (26). There are different approaches in the literature such as using a whole vial of 120 mg, regardless of the infant's weight, or doses of 100 mg/kg or 200 mg/kg (24,25,30). In our experience, we know that reflux of surfactant during LISA is a common issue, experienced by many neonatologists. Due to this knowledge, we believe that following the recommendations of TNS is not only safe, but also offers the extra advantage of delivering the correct amount of surfactant to the lungs. According to results of our study, similar to previous studies, we also showed that the administration of surfactant by LISA is a safe procedure. As LISA is not a common technique, the failure to insert the catheter, a deterioration of the vital signs during the application, a significant surfactant reflux when inserting the catheter to a single bronchus, and/or air leak syndromes are all examples of reported complications (18,19,24,31,32). We did not observe any complications during the process. This may be due to the experience and skill of the dedicated neonatologist/pediatrician performing this procedure as it is one of the most important factors in not experiencing such side effects.

In our study, when we divided the LISA group infants according to their gestational ages, the intubation rates were highest in the <26 gestational-week group and lowest in the 28-29^{6} gestational-week group but this did not reach statistical significance (p=0.617) (Table III). In one paper, where data of the German Neonatal Network was analyzed, it was reported that as the gestational age increased from the 22nd to the 30th weeks, the ratio of the need for mechanical ventilation within the first 3 days decreased (1). We still do not have evidence regarding the possible benefits of LISA for infants over 32 weeks but it is known that these more mature infants may have difficulty in tolerating the procedure without sedation/analgesia. We believe that a study including a larger population and also including >32 week infants will reveal more significant results regarding the sub-group differences relating to intubation needs.

Last but not least, we want to emphasize that LISA must be used as a component of multiple non-invasive/less invasive techniques in order to support the infant's adaptation to the world in a more natural and secure way. LISA should not be applied as an isolated method in order to achieve its maximum benefits. Starting in the antenatal periods, extending to the delivery room and neonatal intensive care units, avoiding all unnecessary procedures and manipulations is important. Otherwise, the LISA technique will not fulfill its potential.

Study Limitations

The main limitation of this study is that we only included infants who were able to survive until their date of evaluation for BPD so we could not make precise analyses about mortality. Another limitation is that medical records of some of the infants could not be accessed. Only one type of surfactant preparation was administered to the infants and no data regarding other types of preparations were available. However, rather than being a limitation, this may even be a positive aspect of our study. As a non-invasive respiratory support modality, we did not further analyze the infants according to mode and both nasal CPAP and nasal SIPPV methods were accepted as a single modality. However, as a standard of care, non-invasive ventilation support was initiated with NCPAP for all infants in the delivery room. The ventilator modality of the infant is chosen as either NCPAP or NSIPPV according to the preference of the attending physician.

Conclusion

The findings of our study have shown that the LISA technique for surfactant delivery to preterms with RDS is a safe method resulting in lower rates of the need for intubation/reintubation. Additionally, in cases where the need for intubation emerged, the intubation duration of those infants was significantly lower in the LISA group. Evaluated at the 36th corrected gestational week, we did not observe any difference in BPD incidences between the two groups. When sub-group analysis was performed according to 3 different gestational ages in the LISA group in order to compare intubation rates, even though differences were present, no statistical significance was observed between the sub-groups.

Ethics

Ethics Committee Approval: This study was approved by Institutional Ethical Committee conducted in Buca Seyfi Demirsoy Training and Research Hospital (approval no: 2021/4-39 dated on 28.04.2021).

Informed Consent: Retrospective, single-center study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: S.G., Concept: S.G., Design: S.G., Data Collection or Processing: S.G., S.Ş., Analysis or Interpretation: S.Ş., Literature Search: S.Ş., Writing: S.Ş.

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Original Article

Dermal Progression of Neonatal Jaundice of Newborn Under 35 Weeks of Gestational Age

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ABSTRACT

Aim: To determine the dermal progression of neonatal jaundice in newborns under 35 weeks of gestational age and those risk factors which affect dermal progression.

Materials and Methods: We prospectively enrolled eighty-eight preterm newborns under 35 weeks of gestational age in neonatol intensive care unit of Dokuz Eylül University Hospital. It was a cross-sectional analytic case study. We measured capillary and transcutaneous bilirubin levels. Multiple sites of TcB measurement were performed.

Results: We observed that there is no significant difference between capillary and transcutaneous bilirubin measurements on preterm newborns under 35 weeks of gestational age (pearson's rho >75 and $p<0.05$). Additionally, we also observed that transcutaneous bilirubin measurements on preterm newborns under 35 weeks of gestational age (the first day taken on the back, the fourth day on the forehead and the remaining days on the chest) are higher than on the other sides (Friedman test). Therefore, for preterm newborns, jaundice progresses in a different way to cephalocaudal direction with progressive hyperbilirubinemia. We did not observe any association between the existence of cephalocaudal progression in preterm newborns and the laboratory data associated with the mother and baby (Mann-Whitney U test, $p>0.05$).

Conclusion: Transcutaneous bilirubin measurements can be used for neonatal jaundice of newborns under 35 weeks of gestational age. However, we need further studies for comprehensive descriptions of preterm newborns' jaundice progression.

Keywords: Dermal progression, neonatal jaundice, gestational age, transcutaneous bilirubin measurements, cephalocaudal progression

Introduction

Neonatal hyperbilirubinemia is the yellow color found in the sclera and skin of infants with increased bilirubin concentration in the plasma. It is one of the most common problems in the neonatal period, being the most frequent cause of hospitalization in the first two weeks of life. The frequency of jaundice is 60% in term and near term infants and 80% in preterm infants in the first week of life, although jaundice requiring treatment is only seen at a rate of 5-6% in newborns.

Neonatal jaundice first becomes visible on the face and forehead, then gradually becomes visible on the trunk and extremities as the level of serum bilirubin rises. This phenomenon is called the "cephalocaudal progression of jaundice". Kramer first described the cephalocaudal progression of jaundice in 1969 (1). Other investigators have confirmed his findings and demonstrated a direct relationship between plasma bilirubin concentrations and the cephalocaudal progression of jaundice (2-4). There are various theories to explain the cephalocaudal progression of jaundice. However, despite its long-time recognition, there has been no satisfactory explanation of how it occurs to date. Kramer suggested exposure to light may play a role (1). Other theories have indicated differences in the epidermis' surface lipid content and albumin's capillary permeability as

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explanations (3). More recently, one theory may explain the cephalocaudal color difference by conformational changes in the bilirubin-albumin complex in the blood (5,6) and differences in the skin temperature and capillary blood flow (7).

Spectrophotometric measurements of the yellow color of the skin and subcutaneous tissues (by transcutaneous bilirubin meter) were introduced in 1980 by Yamanouchi et al. (8) as an alternative to the determination of bilirubin in the serum of neonates.

The exact responsible mechanism for the color of jaundiced skin is unknown. It can show variations due to the skin's natural shade of bilirubin-albumin complexes in the extravascular space and the deposition of bilirubin acid in phospholipid membranes. In a state of equilibrium between plasma and dermal bilirubin concentrations, the intensity of the yellow color skin is related to three factors; plasma bilirubin concentration, the squared hydrogen ion concentration, and the reciprocal of the reverse albumin concentration (9).

In this study, we examined the cephalocaudal progression of jaundice and the effects of clinical and laboratory factors in neonates under 35 weeks of gestational age.

Patients and Methods

This prospective cross-sectional analytical case study was performed between June, 2012 and May, 2013 at the Neonatal Intensive Care Unit (NICU) of Dokuz Eylül University Hospital and it was approved by the Dokuz Eylül University Non-Interventional Research Ethics Committee (date: 17.05.2012, approval no: 2012/18-18). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or the National Research Committee and within the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Due to the prospective nature of this study, informed consent was obtained.

During the study period, newborns under 35 weeks of gestational age were included after parental consent. Eighty-eight newborns admitted to the NICU for various causes were included in this study.

In the inclusion of neonates in this study, the yellow skin color (TcB) measurements were taken at six different sites; forehead (TcBf), sternum (TcBs), abdomen (level of the umbilicus) (TcBa), back (interscapular area) (TcBb), knee (TcBk), and foot (TcBf). TcB was measured daily between the postnatal first and tenth days at these six different sites. We took two readings at each site, and used the average value in the calculation. We did not include those newborns who were already receiving phototherapy or had received phototherapy 24 hours prior to the measurement. We measured TcB with a Minolta Jaundice Mater 103 (Konica Minolta Sensing Inc., Osaka, Japon). TcB color difference measurements (between the highest and lowest value) were made simultaneously on the same day with the same baby.

If we observed jaundice through a blood sample by heel prick, the bilirubin level was determined by a standard direct spectroscopic method using Wako's bilirubin tester.

All values are given as the mean ± standard deviation (Figure 1).

We used the SPSS 17.0 program for statistical analysis. In comparisons between groups, if parametric conditions

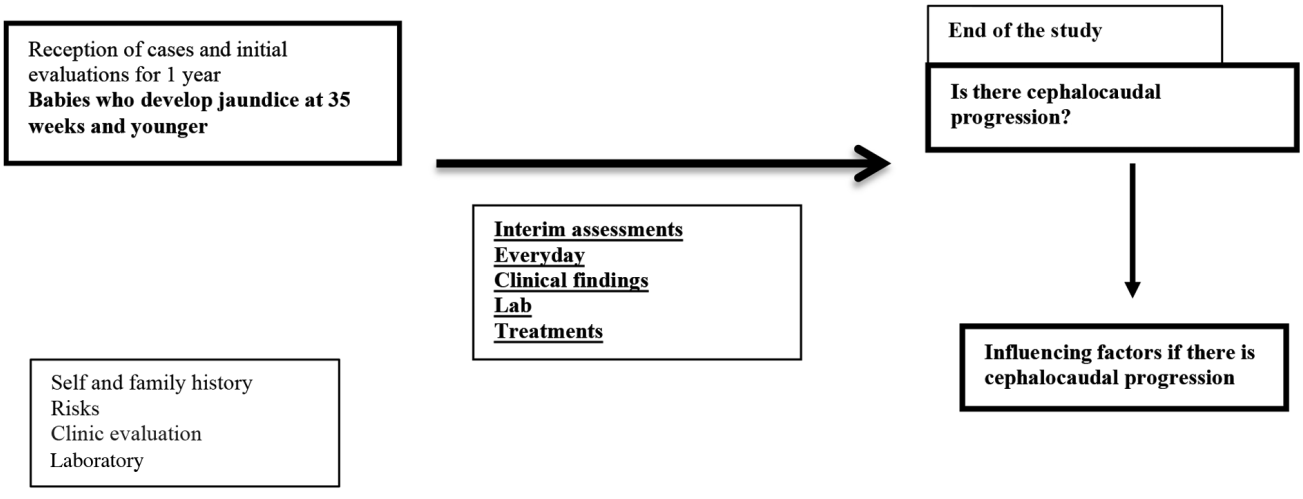

Figure 1. Research design (cross-sectional follow-up and evaluation of infants)

are met, the t-test was employed; if not, the Mann-Whitney U test was used. A statistical significance value of $p<0.05$ was chosen.

Results

Demographic information is given in Table I. The mean gestational age was 30.7±3.3 weeks. The mean gestational weight was 1,617±672 grams. Sixty point two percent of infants were male.

The average and variation values of TcB measurements from the six body sites are shown in Table II. We evaluated TcB measurements from the different body sites with the Friedman test; the highest values were detected on the first day on the back, the fourth day on the forehead, and on the other days on the chest. For each day, the TcB measurement values from different body parts differed, and the ranking in TcB values varied depending on the day, compared by the Friedman test. The highest TcB measurement for each day is marked with an asterisk (*). Day 1 TcB and serum bilirubin measurement were not included in the evaluation because there was no correlation.

The order of TcB measurement values "HIGHEST and LOWEST" from the 6 different body regions for each day are given in Table III.

There was no difference between the ratio of the decrease in TCB values in the knees and the foot and the decrease in the TCB values in the chest 1.-10. between days (Figure 2).

Table IV shows the mean and standard deviation values of the TcB difference variable (the highest-lowest TcB difference for that day) according to the days.

We compared the difference values of all days in pairs with the paired test and applied Bonferroni correction to these results. When the course of the mean of the TcB difference variable according to the days was examined with regression curve estimation models, the averages showed a cubic function, and the model was significant ($p<0.009$) (Figure 3) (When the R2 value of the regression model was examined, it was 0.83, and the explanatory power of the model was 83.7%).

As can be seen in Figure 3, the bilirubin difference persists (Y=5.725X+(-1.037)X2 +0.054 X3).

Concerning the TcB color difference, we reported the highest value on day 3. Regression curve models investigated difference averages by day, and as a result, standards have cubic functions (R^2: 0.83) (Figure 3). When we compared the difference values of all days with the paired test, day three was statistically significant compared to days one, two, and seven. We found the differences between the highest and lowest transcutaneous measurements on the same day in infants continued significantly on day ten, in a similar manner to the other days ($p<0.001$).

The mean value of the difference of TcB in this study (highest measurement-lowest measurement) simultaneously on the same day between the same baby's regions continued meaningfully on day ten, in a similar manner to all other days ($p<0.001$).

Regarding the infants included in this study; 10 (11.4%) had rhesus (Rh) isoimmunization, 3 (3.4%) had ABO blood group incompatibility, 3 (3.4%) had polycythemia, and 1 (1.1%) had a double-volume exchange transfusion. None of the infants had G6PD deficiency, acute bilirubin encephalopathy, cephalohematoma, significant bruising from birth trauma, positive Coombs test, or positive thyroid function tests. We used the hour-specific phototherapy treatment thresholds from the American

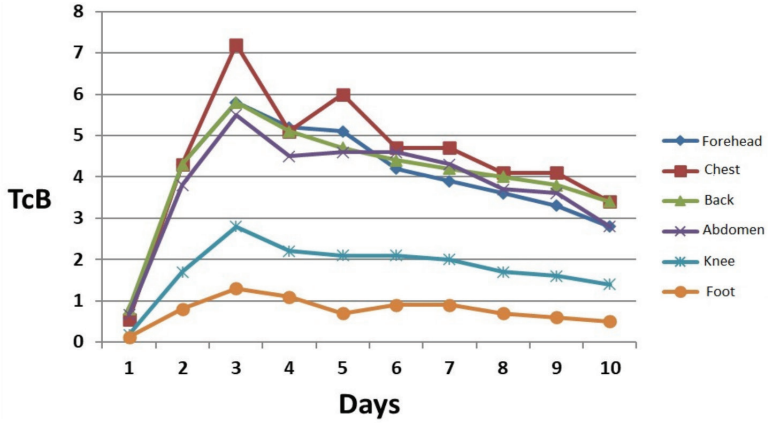

Figure 2. Average values of transcutaneous bilirubin measurements made from six different regions according to days

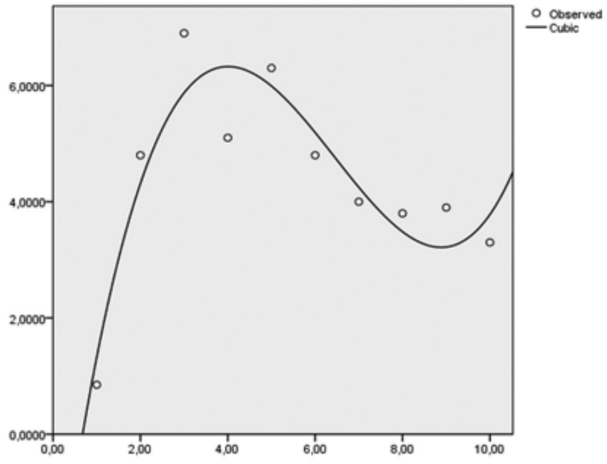

Figure 3. The change in the mean difference of the group of 10 days

Table I. Clinical characteristics of the study group

Features	Number (n=88)	%
Gender		
Male	53	60.2
Female	35	39.8
Birth weight		
1,000>	21	23.9
1,000-1,500	20	22.7
1,500-2,500	40	45.5
+2,500 gr	7	7.7
Average: 1,617±672 gr		
Birth week		
28>	18	20.5
28-32	23	26.1
32-35	47	53.4
Average: 30.7±3.3 week		

Academy of Pediatrics Clinical Practice Guideline on neonatal hyperbilirubinemia management.

We observed no significant difference between capillary and transcutaneous bilirubin measurements (Pearson's rho >75 and p<0.05) (Table V). These findings are consistent with the literature. Studies have shown that TcB and TSB measurements show a perfect correlation (r=0.87-0.96) and a linear relationship between them. In our study, the correlation was weak in the first days with lower bilirubin values. The best correlation was obtained in those measurements from the forehead and chest area.

We evaluated the mean value of the difference of TcB in this study (the highest measurement minus the lowest measurement) on the same day between the same baby's regions non-parametrically by Mann-Whitney U test. It was unrelated to abnormalities in albumin. This is shown in Table VI.

On the 3rd day, the relationship between the average difference and blood gas (with the result of univariate linear regression analysis with blood gas value as the independent variable, and the mean difference on the 3rd day as the result variable), we noted that it was related with blood gas increases. In other words, as the pH increased, the difference increased (p<0.05). However, when we examined the R2 value of the regression model (0.18), it was seen that the explanatory power of the model was low, and this model could only explain 18% of the variation in the difference values. The TcB difference variable was shown not to be affected by plasma albumin level, hematocrit (htc) value, maternal or child disease, Rh incompatibility or ABO incompatibility.

Table II. TcB measurements mean and SD values for daily

Day	n	Forehead (TcBf)	Sternum (TcBs)	Back (TcBb)	Abdomen (TcBa)	Knee (TcBk)	Foot (TcBf)
1	80	0.58±1.4	0.55±1.3	0.8±1.6	0.67±1.6	0.18±0.6	0.12±0.5
2	55	4.3±2.5	4.3±2.8	4.3±2.3	3.8±2.7	1.7±1.7	0.8±1.1
3	44	5.8±8.0	7.2±8.0	5.8±2.4	5.5±2.4	2.8±1.8	1.3±1.5
4	30	5.2±3.0	5.1±3.2	5.1±2.4	4.5±3.0	2.2±2.0	1.1±1.5
5	28	5.1±3.3	6.0±6.1	4.7±2.4	4.6±2.7	2.1±1.8	0.7±1.1
6	39	4.2±2.7	4.7±2.7	4.4±2.4	4.6±2.8	2.1±1.7	0.9±1.2
7	38	3,9±2.9	4.7±3.3	4.2±3.0	4.3±3.3	2.0±1.8	0.9±1.5
8	48	3.6±2.9	4.1±3.2	4.0±3.1	3.7±3.0	1.7±1.8	0.7±1.1
9	44	3.3±3.1	4.1±3.5	3.8±3.0	3.6±3.1	1.6±1.8	0.6±1.4
10	40	2.8±3.0	3.4±3.1	3.4±3.1	2.8±2.9	1.4±1.9	0.5±0.9

SD: Standard deviation

Table III. Ordering of TcB measurements from different body parts by days

Days	
1.	-
2.	Sternum>forehead=back>abdomen>knee>***foot***
3.	Sternum>back=forehead>abdomen>knee>***foot***
4.	***Forehead***>sternum=back>abdomen>knee>***foot***
5.	Sternum>forehead>back>abdomen>knee> ***foot***
6.	Sternum>abdomen>back>forehead>knee>***foot***
7.	Sternum>abdomen>back>forehead>knee >***foot***
8.	Sternum>back>abdomen>forehead>knee>***foot***
9.	Sternum>back>abdomen>forehead>knee>***foot***
10.	Sternum>back>abdomen=forehead>knee>***foot***

Table IV. The difference between the highest and lowest bilirubin values measured transcutaneously

Days	TcB difference variable (Mean±SD)
1	0.85±1.6
2	4.8±2.3
3	6.9±7.8
4	5.1±2.5
5	6.3±5.7
6	4.8±2.4
7	4.0±2.7
8	3.8±2.6
9	3.9±2.9
10	3.3±2.6

SD: Standard deviation

Table V. Correlation levels between TcB measurements from the six different body regions and TSB measurements

Forehead (TcBh)	0.95***
Sternum (TcBs)	0.96***
Back (TcBb)	0.86***
Abdomen (TcBa)	0.85***
Knee (TcBk)	0.72**
Foot (TcBf)	0.74**

*Rho 25-50 and p<0.05, **Rho 50-75 and p<0.05, ***Rho >75 and p<0.05

Table VI. Comparison of transcutaneous bilirubin difference values according to albumin value groups

Albumin	3rd day difference average	
	Median (min.-max.)	**p***
Normal (n=20)	6.2 (1.7-9.1)	0.196
Low (n=5)	5.5 (3.4-6.6)	

Min.-max.: Minimum-maximum

These current findings suggest that the course of jaundice in neonates younger than 35 weeks was differentially centrifugal.

Discussion

Knudsen and Ebbesen (6) investigated the cephalocaudal progression of jaundice in 377 newborn babies. They made two transcutaneous measurements on the forehead, sternum, knee, and foot regions with a JM-101 device and evaluated the accompanying clinical and laboratory factors (6). Knudsen and Brodersen (9) suggested that bilirubin is transferred to the skin by two different mechanisms. The first of these pathways is the transition of bilirubin-albumin complexes from the plasma to the extravascular compartment. The second is the precipitation of bilirubin acid in phospholipid membranes. The bilirubin acid supersaturates the plasma of the newborn. *In vitro* studies have shown that pigment precipitates immediately on phospholipid membranes in contact with supersaturated bilirubin solution.

Bilirubin, which is present as a dianion in the bilirubin-albumin complex, combines with two hydrogen ions in the plasma, after which solid bilirubin acid accumulates on the capillary wall, and one molecule of albumin is released into the plasma (4-6). Various hypotheses have tried to explain the cephalocaudal progression of bilirubin; namely, regional skin vascularity differences, regional differences in epidermal lipid content, variations in skin temperature, capillary blood flow, and Knudsen's bilirubin-albumin binding time. The publications of Knudsen and Brodersen (9) cover young albumin-bilirubin complexes circulating in the blood and their conformational changes over time. In this study, the young complexes were separated and extravasated easily, and a tight connection occurred between the complex over time. More youthful complexes in proximal body parts are associated with cephalocaudal progression. The theory is that cephalocaudal progression increases with bilirubin concentration and decreases with albumin's affinity for bilirubin. However, the time for complexes to reach the most distal parts of the body is shorter than the tight junction formation time (the tight junction formation time between albumin and bilirubin starts in 30 seconds and ends in about 8 minutes. However, the blood travel time from the aorta to the foot in a newborn is 4.3 seconds, and blood travels at 1.1 m/sec. In other words, albumin-bilirubin complexes arrive at the foot without having formed a tight connection) (4-9,10).

In our study, we found transcutaneous bilirubin measurements to be higher on the back on the first day, on the forehead on the fourth day, and on the chest on the other days in preterms under 35 weeks during the first ten days. Our study shows that, unlike term babies, the most significant elevation is on the chest region in transcutaneous bilirubin measurements in preterms under 35 weeks, and that jaundice progression follows a different spread, i.e. not from head to toe as in term babies. In this respect, our findings are not similar to the data in the literature (4,6). Our study suggests that jaundice progression in preterms under 35 weeks of age follows a different "centrifugal" spread, not from head to toe as in term babies. In our study, the course of jaundice in preterms may be associated with local factors, such as the lipid content of the skin, the basal skin color of the baby, differences in blood flow, differences in permeability of regional capillaries to albumin, skin perfusion and temperature, decreased capillary flow in the distal skin regions, increased bilirubin production, decreased bilirubin removal from the blood, and/or increased enterohepatic circulation (4,6,7).

TcB measurements on the chest area correlated very well with TSB measurements, suggesting that it would be appropriate to measure TcB on the chest area.

Whether the mother has a history of hypertension, preeclampsia, gestational DM, Rh incompatibility, or antenatal steroid use during pregnancy and whether the patient has a history of direct Coombs negativity, polycythemia, abnormalities in hemogram, biochemistry, or albumin levels, we analyzed the distribution in terms of transcutaneous bilirubin measurement differences, and no difference was found. No similar study was found in the literature (11,12-24). The change in bilirubin difference significantly affects the increase in blood gas. In other words, the difference increases as the pH increases (p-value<0.05). These results are consistent with the literature (5).

We found a statistically significant correlation between transcutaneous-capillary bilirubin measurements. We have shown that using the TcB measurement as a screening tool to determine the necessity of serum bilirubin measurement is reliable for preterm infants. Many studies in the literature have also shown that there is a correlation between TcB and TSB measurements (8,10-23).

Study Limitations

This study has some limitations. The most important limitation is the small sample size. Additionally, it is also possible that the cephalocaudal progression of icterus spreads slower in neonates born closer to term than our population of preterms under 35 weeks of gestational age, as seen in the population of the study of Kamphuis and Bekhof (25).

More studies are needed to better understand the dermal kinetics of bilirubin. A better understanding of bilirubin kinetics may offer new possibilities for preventing bilirubin encephalopathy.

Conclusion

We found a statistically significant correlation between transcutaneous-capillary bilirubin measurements. We have shown that using the TcB measurement as a screening tool to determine the necessity of serum bilirubin measurement is reliable for preterm infants. Many studies in the literature have also shown that there is a correlation between TcB and TSB measurements. And also, our study shows that, unlike term babies, the most significant elevation is on the chest region in transcutaneous bilirubin measurements in preterms under 35 weeks and that jaundice progression follows a different spread, not from head to toe as in term babies. In this respect, our findings are not similar to the data in the literature. Our study suggests that jaundice progression in preterms under 35 weeks of age follows a different "centrifugal" spread, not from head to toe as in term babies.

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Ethics

Ethics Committee Approval: This study was approved by the Dokuz Eylül University Non-Interventional Research Ethics Committee (date: 17.05.2012, approval no: 2012/18-18).

Informed Consent: Informed consent was obtained.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Ç.Ç.K., B.İ., A.K., Concept: Ç.Ç.K., N.D., H.Ö., Design: Ç.Ç.K., H.Ö., A.K., Data Collection and/or Processing: B.İ., A.K., H.Ö., Analysis and/or Interpretation: B.İ., N.D., H.Ö., Literature Search: B.İ., N.D., H.Ö., A.K., Writing: Ç.Ç.K.

Conflict of Interest: All of the authors declare that they have no conflict of interest.

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Original Article

Impact of a Multi-Strain Probiotic on Healthcare-Associated Bloodstream Infection Incidence and Severity in Preterm Neonates

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ABSTRACT

Aim: Hospital acquired bloodstream infection (HA-BSI) is a major contributor to morbidity and mortality in preterm, very low birthweight infants, especially in low-to-middle- income countries.

Materials and Methods: We conducted a double-blind, placebo-controlled, randomized clinical trial to investigate the effect of a multi-strain probiotic formulation (LabinicTM) on the incidence and severity of HA-BSI in preterm neonates.

Results: Two hundred neonates (100 per arm) were included in this trial. Fifteen neonates developed HA-BSI events (2 in the probiotic arm and 13 in the placebo arm). The median day of life at HA-BSI onset for the probiotic group was 10.5±3.5, and for the placebo group, it was 11.2±6.4. The incidence of HA-BSI in neonates receiving the probiotic was significantly lower compared to those receiving the placebo [0.93 versus 5.99 HA-BSI events/1,000 neonate-days; incidence rate ratio (IRR) of 0.156 [95% confidence interval (CI): 0.017 to 0.691], p=0.0046]. Calculating the incidence rate of the combined outcome (sepsis/death) was also lower in the probiotic group versus the placebo group [2.34 versus 6.45 events/1,000 neonate days; IRR 0.33 (95% CI: 0.11 to 0.97), p=0.043].

Conclusion: The use of a multi-strain probiotic significantly reduced HA-BSI incidence in this cohort of preterm neonates.

Keywords: Healthcare-associated bloodstream infection, neonate, probiotic

Introduction

Globally, neonatal infections cause an estimated 26% of all neonatal deaths, with the highest infection-related mortality observed in Sub-Saharan Africa (1,2). Hospital acquired bloodstream infection (HA-BSI), defined as BSI occurring 48-72 hours after birth, are the most frequent infection type encountered in hospitalised neonates (3). The incidence of HA-BSI is inversely related to neonatal gestational age and birth weight with preterm (<37 weeks gestation) and very low birth weight neonates (<1,500 g) at particularly elevated risk (4).

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Dong and Speer (3) analysed data from 11 studies globally concluding that extremely preterm (<28 weeks' gestation) versus late preterm neonates (33-36 weeks' gestation) had a two-fold higher HA-BSI prevalence (36% vs 18%). In South African hospitals, approximately 1 in 10 preterm neonates develop HA-BSI. Lebea (5) found an incidence rate of blood-culture confirmed neonatal sepsis of 10.3 per 100 admissions, with HA-BSI accounting for 83.7% of all BSI epsiodes. Similar results were found by Motara et al. (6) with 8.1% of hospitalised neonates developing HA-BSI. In Sub-Saharan Africa, gram-negative pathogens cause two-thirds of neonatal HA-BSI and are often multidrug-resistant with substantially higher mortality rates than those observed for Gram-positive pathogens (7,8).

Neonates with a birth weight <1,500 g show delayed intestinal colonisation with normal microbial flora (3). Contributing factors include birth by caesarean section, lengthy antibiotic use, use of infant formula and parenteral nutrition, delayed skin contact and sub-optimal infection prevention practices in hospital. This abnormal gut flora (dysbiosis) can lead to bacterial translocation and HA-BSI in preterm neonates (9). An additional risk factor for HA-BSI includes the presence of invasive or indwelling devices. Important gastrointestinal risk factors for HAI include immature mucosal gut barriers, intestinal ischemia, hyperosmolar injury, bacterial invasion, and subsequent inflammation (10).

The human gut microbiome plays a pivotal role in nutritional, physiological, immunological, and protective functions (11). However, the neonatal gut microbiome may be altered, or have delayed maturation following preterm birth, antibiotic administration, and/or delays in establishing enteral feeds (12). These factors reduce the activity of beneficial bacteria e.g., *Lactobacilli* and *Bifidobacteria* and promote overgrowth of pathogenic gut bacteria, resulting in bacterial translocation and the potential to develop HA-BSI (13).

Providing probiotic therapy to preterm neonates may promote intestinal colonisation with normal, beneficial microbial flora and prevent the overgrowth of pathogens (7). The putative mechanisms of BSI prevention through probiotic supplementation include modulation of immune response parameters with increased anti-inflammatory cytokine production and stabilization of the gut barrier function, with improved intestinal integrity and colonization resistance (14,15). The effect of probiotic supplements is enhanced in neonates receiving breastmilk feeds, possibly owing to breastmilk-induced reductions in gut permeability to pathogen translocation and anti-infective components such as lactoferrin, IgA, IgG, IgM and oligosaccharides, which act synergistically as a prebiotic (8).

In a systematic review and meta-analysis, Dermyshi et al. (16) showed that probiotics reduced neonatal sepsis rates by 12% and 19% (pooled data from randomized controlled trials and observational studies, respectively). They concluded that the use of the *Lactobacillus* species or a mixture of 2-3 species of bacteria might be the most efficacious (16). Although the modest benefits of probiotics for HA-BSI prevention are promising, the optimal microbial strains, combinations, dosing, timing and duration of supplementation, and their efficacy in neonates has not been definitively elucidated. We aimed to determine whether the administration of a multi-strain probiotic could reduce the incidence and severity of HA-BSI in African neonates.

Materials and Methods

Study Design

We conducted a double-blind, placebo-controlled, randomized clinical trial to investigate the effect of a multi-strain probiotic formulation on the incidence and severity of HA-BSI in preterm neonates. This manuscript was prepared in accordance with the CONSORT statement checklist for the reporting of clinical trials.

Study Setting

Tygerberg Hospital (TBH) situated in Cape Town, South Africa, is a tertiary hospital with 1,384 beds, serving the Cape Metro Region's Northern and Eastern sub-districts and the surrounding rural districts' healthcare facilities. The neonatal unit inside TBH consists of 132 beds, including a 12-bed medical/surgical neonatal intensive care unit, 2 high-dependency wards, 1 low-care ward and 1 kangaroo mother care ward. Study participants were recruited from the two high-dependency neonatal wards. Participants were enrolled between the 19th January and 27th June, 2021.

Study Participants

Preterm neonates aged 1-3 days of life at enrolment, with a birth weight between 750-1,500 grams and <37 weeks' gestation were eligible for inclusion. Exclusion criteria were severe or life-threatening congenital anomalies, early onset neonatal sepsis [C-reactive protein (CRP) >10 mg/L in the first 72 hours of life], (17) neonates scheduled for adoption, major gastro-intestinal abnormalities, or surgery of the gastro-intestinal tract. This study had four main outcomes, of which HA-BSI compromised one. We used one of the

other study outcomes, namely a reduction in the carriage rate of antibiotic resistant organisms, to calculate the required sample size for this study. A proportion difference of a 17% decrease in rectal colonisation with drug-resistant bacteria was used to estimate the sample size required to detect a significant difference between the groups being compared (with a Type I error of 0.05 and a power of 80%). The total sample size required was 200 neonates or 100 per group (allowing for a 12% margin for study participants lost-to-follow-up).

Randomisation

A pre-determined randomization list prepared by the study statistician was used to randomly allocate neonates to the two balanced study arms (n=100 each) - a probiotic (intervention) group and a placebo group. Consecutive sampling was used i.e., every preterm neonate meeting the inclusion criteria was selected until the required sample size was achieved.

The manufacturer packaged the products (probiotic or placebo) and did the allocation concealment. The packaging of the two products was identical apart from a distinguishing pink or green sticker. Once enrolled, each neonate received their own probiotic or placebo bottle in order to avoid contamination and to ensure that the infant received the same treatment over time. The researcher and all neonatology staff were blinded as to which of the two groups received the probiotic versus the placebo.

Procedures

A multi-strain probiotic containing *Lactobacillus acidophilus* [0.67 billion colony forming units (CFU)s], *Bifidobacterium bifidum* (0.67 CFUs) and *Bifidobacterium infantis* (0.67 CFUs) was used, Labinic™ (Biofloratech, Surrey, United Kingdom). The placebo consisted of medium chain triglyceride oil and Aerosil 200 (Aerosil 200 is the stabiliser also used in Labinic™).

The standard dose of 0.2 mL was administered, providing 2 billion CFUs per day. Supplementation with the probiotic or placebo was delayed if the neonate was *nil per os* and discontinued if a neonate developed necrotizing enterocolitis (NEC) (Bells stage II or more) (18). The researcher added the probiotic/placebo to the neonate's feed (mother's own breast milk/donor breast milk/neonate formula) before administration of the feed via an orogastric tube or if applicable, orally. The probiotic/placebo was administered once daily to the neonate's morning feed and the neonates were followed up from birth to a maximum of 28 days of life, death, discharge to peripheral hospitals or home, depending on whichever time-point came first.

Data collected at enrolment included neonatal demographic information, estimated gestational age (early/late ultrasound or foetal foot length), gender, birth weight, type of delivery, ethnicity and Apgar scores. Daily data collected included reviewing the clinical notes, laboratory records, anthropometric measurements, recording the type and volume of feeds received, infections present (e.g., meningitis, urinary tract infection, pneumonia, tuberculosis) and any medication prescribed.

HA-BSI was defined as a positive blood culture with a known neonatal pathogen obtained after 72 hours of life together with a CRP above 10 mg/L (19). HA-BSI was excluded in the presence of a negative blood-culture and/or a CRP<10 mg/L. Central line associated bloodstream infections are not part of HA-BSI and were not part of the protocol. Organisms were classified using the United States Centers for Disease Control list of pathogens and contaminants (https://www.cdc.gov/hai/organisms/organisms.html). Repeat blood cultures isolating the same pathogen within 10 days of the original specimen were considered to represent a single episode of infection. VLBW infants with blood cultures isolating known skin commensals or contaminants were excluded from further study end point analysis (20). A poly-microbial infection was defined as the isolation of more than one pathogenic organism from a single blood culture.

Hospital guidelines recommend routine blood culture collection at birth for neonates with obstetric risk factors for infection e.g., prolonged rupture of membranes, chorioamnionitis, or suspected sepsis. Neonates who develop clinical signs and symptoms of infection during hospital admission also undergo a sepsis work-up including full blood count, CRP and blood culture collection as minimum laboratory investigations. Approximately 1-2 mL aseptically collected blood is inoculated into a paediatric blood culture bottle (BacT/ALERT PF bottle) and submitted to the on-site National Health Laboratory Services (NHLS) which uses the automated BacT/Alert blood culture system (BioMerieux, Marcy l'Etoile, France). If bacterial growth is detected, a Gram stain is performed and the sample sub-cultured onto appropriate media and incubated overnight. Further identification and antimicrobial susceptibility testing of clinically significant isolates is performed with the automated Vitek II system (BioMerieux) using Clinical and Laboratory Standards Institute breakpoints. If urinary

tract infection, meningitis or another infection focus is suspected, additional laboratory specimens are submitted.

In most instances, the following antibiotics are used (local hospital guidelines): ampicillin and gentamicin if the neonate is <72 hours of life; if the neonate is ≥72 hours of life, piperacillin-tazobactam plus amikacin is used for stable neonates, and meropenem for critically ill neonates or neonates with suspected meningitis. Neonates with HA-BSI in the presence of thrombophlebitis or the recent use of central lines have vancomycin added to their antibiotic treatment at the clinician's discretion. Following pathogen identification and antibiotic susceptibility testing, the empiric antibiotic regimen is adapted to provide the narrowest spectrum treatment possible or discontinued if the blood culture is negative.

Statistical Analysis

A baseline table of demographic and clinical characteristics were tabulated by group and contains frequencies, percentages, and medians. An intention to treat analysis comparing the probiotic vs placebo arms for HA-BSI incidence was performed. The HA-BSI incidence rates, calculated using the total number HA-BSI events in each trial arm divided by the respective neonate days x 1,000, were used to calculate the incidence rate ratio (IRR) with 95% confidence intervals (CI). A sensitivity analysis was conducted using a Poisson regression model to estimate the IRR adjusted for some baseline factors: gender, maternal age, birthweight, gestational age, and day of commencement of enteral feeds. The proportion of neonates receiving antibiotics in each group were compared using a chi-squared test. For all statistical tests performed, a p-value <0.05 was considered significant. All the statistical analyses were performed using STATA 16.0 (College Station, Texas 77845 USA).

Ethical Approval

Ethical approval was granted by the Health Research Ethics Committee of the Faculty of Health Sciences of Stellenbosch University as well as Tygerberg Hospital (S20/07/178). This trial was registered in the Pan African Clinical Trial Registry (PACTR202011513390736). Written informed consent was obtained from each neonate's mother.

Role of the Funding Source

The funders of this trial had no role in trial design, data collection, data analysis, data interpretation, or the writing of this report.

Results

A total of 709 neonates were screened for this study, of which 313 were eligible for inclusion. Of these neonates, 207 were enrolled, but 7 neonates developed early complications prior to receiving the placebo/probiotic and they were excluded from subsequent analysis. Two hundred neonates (100 per arm) were included in this trial (Figure 1). Of the 200 enrolled neonates, 100 (50%) completed the full 28-day study period in the neonatal unit and the remainder were either transferred to other hospitals (47%), discharged (0.5%) or passed away (2.5%). The mean number of days enrolled in this study was similar between the two groups: probiotic 21.35 (±7.69) days and placebo 21.70 (±7.62) days.

The participants' mean gestational age was 29 weeks ±13.9 days (range 25-36 weeks), in the probiotic group and 30 weeks ±13.5 days (range 25-34 weeks) in the placebo group. The participants' mean birth weight was 1,174 g ±226 g (range 780 g-1,500 g) in the probiotic group and 1,150 g ±230 g (range 750 g-1,495 g) in the placebo group. Nearly a quarter of the neonates (23%) were HIV-exposed, but none returned a positive HIV PCR test at birth (Table I). The mode of delivery did not differ between the two groups and nearly three out of four neonates (73%) were delivered by caesarean section (Table II).

The mean day of life at HA-BSI onset for the probiotic group was 10.5±3.54, (range 8-10 days) and for the placebo

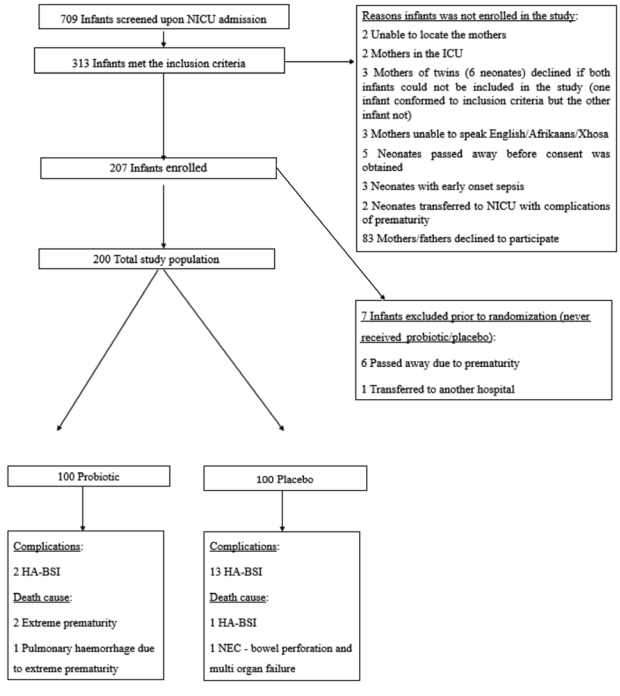

Figure 1. Flow diagram of neonates included in the clinical trial

Table I. Demographic and clinical characteristics of neonates enrolled in the study (n=200)

	Probiotic group (n=100)	Placebo group (n=100)
Gender		
Male (n, %)	47 (47)	37 (37)
Female (n, %)	53 (53)	63 (63)
Birth weight		
750-1000 g (n, %)	30 (30)	32 (32)
1001-1500 g (n, %)	70 (70)	68 (68)
Gestational age		
26-28 weeks (n, %)	34 (34)	30 (30)
29-32 weeks (n, %)	60 (60)	62 (62)
33-36 weeks (n, %)	6 (6)	8 (8)
Apgar score (10 min)		
<4 (n, %)	0 (0)	1 (1)
4-7 (n, %)	10 (10)	9 (9)
>7 (n, %)	89 (89)	89 (89)
No Apgar (born before arrival (n, %)	1 (1)	1 (1)
HIV		
Exposed (n, %)	22 (22)	26 (26)
Unexposed (n, %)	78 (78)	74 (74)
First feed received		
EBM (n, %)	68 (68)	69 (69)
DEBM (n, %)	12 (12)	6 (6)
PEBM (n, %)	19 (19)	25 (25)
FM (n, %)	1 (1)	0 (0)
Subsequent feeds received*		
EBM (n, %)	63 (63)	66 (66)
DEBM (n, %)	13 (13)	9 (9)
PEBM (n, %)	15 (15)	24 (24)
FM (n, %)	9 (9)	1 (1)

*The feed received most often (>50% of the time)
DEBM: Donor expressed breastmilk, EBM: Expressed breastmilk, FM: Formula milk, PEBM: Pasteurized expressed breastmilk

group, it was 11.15±6.37, (range 4-28 days). The incidence of HA-BSI in the probiotic arm was significantly lower compared to those receiving the placebo (Table III). HA-BSI episodes occurred in 2 (2%) neonates receiving the probiotic and in 13 (13%) neonates in the placebo group. The incidence of HA-BSI in neonates receiving the probiotic was significantly lower compared to those receiving the placebo [0.93 versus 5.99 HA-BSI events/1,000 neonate-days; IRR of 0.156 (95% CI: 0.017 to 0.691), p=0.0046], using total neonatal study days as the denominator (2,135 days in the probiotic group and 2,170 days in the placebo group). *Klebsiella pneumoniae* was cultured in 2/2 (100%) of the neonates in the probiotic group. The organisms cultured in the placebo group varied, with the main organisms being *Serratia marcescens* 4/15 (31%), *Klebsiella pneumoniae* 3/15 (23%) and *Enterococcus faecalis* 3/15 (23%) (Table IV).

Table II. Delivery information of the mothers (n=200)

	Probiotic group (n=100)	Placebo group (n=100)
Maternal age		
18-20 years (n, %)	16 (16)	13 (13)
21-30 years (n, %)	41 (41)	52 (52)
31-40 years (n, %)	39 (39)	32 (32)
41-45 years (n, %)	4 (4)	3 (3)
Mode of delivery		
C-section (n, %)	73 (73)	73 (73)
Vaginal delivery (n, %)	27 (27)	27 (27)
Maternal steroids		
Received (n, %)	84 (84)	89 (89)
Birth number		
Single neonate (n, %)	79 (79)	86 (86)
Twin neonates (n, %)	21 (21)	14 (14)
Reason for premature delivery		
SPPROM (n, %)	16 (16)	20 (20)
FD (n, %)	57 (57)	43 (43)
EOPET (n, %)	2 (2)	4 (4)
Placenta abruption (n, %)	2 (2)	7 (7)
IUGR (n, %)	1 (1)	6 (6)
SPTL (n, %)	18 (18)	18 (18)
HELLP (n, %)	2 (2)	1 (1)
Placenta praevia (n, %)	2 (2)	1 (1)

EOPET: Early onset pre-eclampsia, FD: Foetal distress, HELLP: Haemolysis, elevated liver enzymes, low platelet count, IUGR: Intrauterine growth restriction, SPPROM: Spontaneous preterm premature rupture of the membranes, SPTL: Spontaneous preterm labour

Gender, maternal age, birth weight, gestation as well as day of starting enteral feeds were not significant covariates. Adjusting for baseline covariates, the probiotic effect of preventing sepsis showed an IRR of 0.134 (95% CI: 0.028-0.642), p=0.012.

The percentage of neonates who received empiric antibiotic therapy at birth for possible infection was similar between the two groups [placebo, n=54 (54%)

Table III. Clinical data of the neonates (n=200)		
	Probiotic group (n=100)	**Placebo group (n=100)**
Invasive interventions		
Nasogastric tube inserted days (mean; ±SD)	20.9±7.8 (range 2-28)	21.4±7.8 (range 7-28)
TPN line inserted days (mean; ±SD)	0.1; ±1.00 days (range 0-8)	0.5; ±2.2 (range 0-15)
IV-line inserted days (mean; ±SD)	8.1±2.4 (range 4-21)	8.9±4.2 (range 5-28)
Mechanical ventilated days (mean; ±SD)	0	0.1±0.9 (range 0-7)
CPAP days (mean; ±SD)	5.2±4.9 (range 0-26)	5.5±5.2 (range 0-28)
High flow days (mean; ±SD)	3.3±4.9 (range 0-21)	3.7±4.0 (range 0-20)
Nasal prongs days (mean; ±SD)	3.8±5.1 days (range 0-22)	4.3±5.0 (range 0-24)
Day on which feeds was initiated		
DOL (mean days; ± SD)	3.1±1.1 (range 0-6)	3.0±1.0, (range 2-6)
Days to achieve full feeds		
DOL (mean days; ±SD)	8.7 ±2.0 (range 5-18 days)	9.7±4.3, (range 6-28 days)
Number of days on TPN (mean days, +- SD)	0.1 days; ±1.0 (range 0-8)	0.5 days; ±2.2 (range 0-15)
Number of days NPO (mean days, +- SD)	0.2 days; ±0.4 (range 0-8)	0.4 days; ±0.7 (range 0-8)
Neonates classified as at septic risk at birth and received empiric antibiotics	57	55
Empiric antibiotic use for presumed sepsis at birth (n) Days (mean days; ±SD)	57 3.8; ±2.1, (range 1-12)	55 3.8; ±2.0, (range 1-10)
Empiric antibiotic regimens (<72 hours of life) (n; %) ampicillin plus gentamicin piperacillin-tazobactam plus amikacin vancomycin meropenem	 52 (91) 12 (21) 1 (2) 1 (2)	 53 (96) 5 (9) 0 (0) 3 (6)
Positive cultures		
Number of blood cultures submitted	89	119
Neonates with positive blood culture (n, %)	2 (2)	13 (13)
Total number of pathogens isolated from the cultures requested as per above (n=23)	2	16
Monomicrobial BSI	2	10
Polymicrobial BSI	0	3
Day of life at HA-BSI onset, (mean±SD)	10.5±3.5, (range 8-10)	11.2±6.4, (range 4-28)
Targeted antibiotic regimens used for HA-BSI episodes after blood culture results were available	n=2	n=13
Neonates that received the antibiotic (n; %) piperacillin-tazobactam plus amikacin meropenem plus vancomycin ampicillin plus gentamicin meropenem plus colistin meropenem	 0 (0) 0 (0) 0 (0) 0 (0) 2 (100)	 4 (26.5) 2 (13) 3 (23) 3 (23) 3 (23)
Infants that developed HA-BSI	n=2	n=13
Weight		
750-1000 g (n, %)	2 (2%)	8 (8%)
1001-1500 g (n, %)	0 (0%)	5 (5%)
Gestational age		
26-28 weeks (n, %)	0 (0%)	8 (8%)
29-32 weeks (n, %)	2 (2%)	5 (5%)
33-36 weeks (n, %)	0 (0%)	0 (0%)

CPAP: Continuous positive airway pressure, DOL: Day of life, NPO: Nul per os, TPN: Total parenteral nutrition, HA-BSI: Hospital acquired bloodstream infection, SD: Standard deviation

versus probiotic, n=57 (57%)]. When analysing the subset of neonates who received empiric antibiotics and later developed HA-BSI, there was no significant difference in the occurrence of sepsis between the two groups, with sepsis occurring in 3.5% of the probiotic group (2/57) neonates, and 7.3% (4/55 neonates) in the placebo group (IRR: 0.467, p=0.4064). The probability of empiric antibiotic use in the probiotic group was 1.09 times as high as for the placebo group but not significantly different (p=0.788).

HA-BSI incidence rates were higher among neonates who did not received antibiotics at birth, compared to those who had (9/88 vs 6/112; p=0.194). In the sub-group considered not at risk of sepsis at birth, with no empiric antibiotic use, 0% (0/43) of these infants developed sepsis in the probiotic group, versus 20% (9/45) in the placebo group (p<0.004).

Five neonates passed away, 2 in the placebo group passed away (HA-BSI on day 8 of life, NEC on day 21 of life) and 3 in the probiotic group (2 from extreme prematurity on day 7 of life, and one from pulmonary haemorrhage on day 15 of life). There was a significant risk reduction in survival for neonates in the probiotic group. The incidence rate of the combined outcome (sepsis/death) was lower in the probiotic group versus the placebo group [2.34 versus 6.45 events/1,000 neonate days; IRR 0.33 (95% CI: 0.11 to 0.97), p=0.043].

In calculating the sepsis/death incidence rate per 1,000 neonate-days, there were 2.34 events in the probiotic group versus 6.45 in the placebo group. The IRR of probiotic relative to placebo sepsis/death events was 0.33 (95% CI: 0.11 to 0.97), p=0.043.

Other infection types that were documented during the trial included: urinary tract infection (1, placebo group), congenital tuberculosis (1, probiotic group), and pneumonia (3 in the probiotic and 1 in the placebo group).

No protocol violations nor serious adverse events relating to the use of the probiotic occurred.

Table IV. Neonatal HA-BSI pathogens

	Probiotic group (n=2) n (%)	**Placebo group (n=16*) n (%)**
Organisms isolated		
Klebsiella pneumoniae	2 (100%)	3 (19%)
Serratia marcescens	0 (0%)	4 (25%)
Enterococcus faecalis	0 (0%)	3 (19%)
Staphylococcus aureus	0 (0%)	2 (12.5%)
Acinetobacter baumannii	0 (0%)	2 (12.5%)
Klebsiella oxytoca	0 (0%)	1 (6%)
Proteus mirabilis	0 (0%)	1 (6%)

HA-BSI: Hospital acquired bloodstream infection
*(10 Infants had a monomicrobial BSI, and 2 infants had a polymicrobial BSI).

Discussion

HA-BSI is a leading cause of illness and death in hospitalised preterm neonates in South Africa (5,6,21). South African data shows that around 10% of preterm neonates develop HAI (5). In our study, the probiotic group showed an 84% risk reduction in the incidence of HA-BSI, compared to the placebo group when a multi-strain probiotic, Labinic™, was administered daily for a duration of up to 28 days. A review of previous probiotic studies confirms that multi-strain probiotics are preferable to single-strain probiotics, as they were more likely to be associated with a statistically significant reduction in HA-BSI rates and/or death. A systematic review and meta-analysis by Dermyshi et al. (16) in 2017 recommended that a multi-strain probiotic containing *Lactobacillus acidophilus* together with *Bifidobacterium infantis* or others should be considered. Their analysis showed that single-strain probiotics e.g., *Lactobacillus reuteri*, *Bifidobacterium breve* or *Saccharomyces boulardii,* had no effect in reducing HA-BSI or mortality (16). Kanic et al. (22) also showed a statistically significant reduction in HAI when using a multi-strain probiotic containing *Lactobacillus acidophilus* (*subsp. Lactobacillus Gasseri), Bifidobacterium infantis* and *Enterococcus faecium*. Unfortunately, a large prospective trial (the Proprem trial) using *Bifidobacterium lactis, Streptococcus thermophilus* and *Bifidobacterium infantis* showed no significant reduction in sepsis or mortality (23). At the same time, most single-strain trials failed to show any beneficial effects. A randomized controlled trial by Mihatsch et al. (24) showed that a single strain of *Bifidobacterium lactis* did not reduce the incidence of nosocomial infections in VLBW infants. A multicentre trial by Dani et al. (25) using *Lactobacillus rhamnosis* also showed no significant reduction in bacterial sepsis compared to a placebo.

The diagnosis of HAI may be difficult to confirm and thus empiric antibiotic therapy is promptly initiated for neonates at high-risk of infection e.g., prolonged rupture of membranes, or chorioamnionitis (26). The use of empiric antibiotics in our study was similar between the probiotic and placebo groups (3.75 days vs 3.80 days). However, the use of antibiotics for a confirmed HA-BSI differed between the probiotic and placebo groups (8.40 days vs 11.64 days).

In the subgroup of neonates not classified as at septic risk with no empiric antibiotic use, there was a large difference in the occurrence of HA-BSI with 0% detected in the probiotic group (0/43) vs 20% (9/45) in the placebo group ($p<0.004$). The probiotic intervention thus especially protected those neonates who did not receive empiric antibiotics.

Klebsiella pneumoniae was identified in the blood culture of both neonates who developed HA-BSI in the probiotic group. The main organisms identified in the placebo group were *Serratia marcescens*, *Klebsiella pneumoniae* and *Enterococcus faecalis.* A previous study by Dramowski et al. (27) at the same institute identified *Klebsiella pneumoniae* and *Staphylococcus aureus* as the leading neonatal pathogens. In keeping with other studies on neonatal HAI, *Serratia marcescens* was a major contributor to HA-BSI events in our trial cohort (28,29).

A limitation of this study was the high proportion of the study population who were transferred out to peripheral hospitals, owing to high occupancy rates at the tertiary hospital, which led to reduced days of observation during this trial. The NHLS database was screened for all study participants who were transferred to peripheral hospitals for subsequent blood cultures. None of the infants yielded a positive blood culture up until day 28 of life.

Besides the morbidity and mortality associated with HA-BSI, it has been shown that inflammation can also contribute to long-term neuro developmental impairment as it adversely affects the preterm brain (30).

The use of a multi-strain probiotic shows great potential as a cost effective and safe method of reducing HA-BSI and subsequent mortality in preterm neonates. Probiotics are potentially the most cost-effective intervention for the prevention of HA-BSI. As Athalye-Jape and Patole (31) concluded, no intervention comes close to probiotics in the reduction of length of stay at a cost of less than a dollar per day. Multi-strain probiotics (through reduction in HA-BSI events) could potentially reduce the length of hospital stay in preterm neonates and thus be a resource and cost saving intervention. This study showed that a multi-strain probiotic (*Lactobacillus acidophilus*, *Bifidobacterium bifidum* and *Bifidobacterium infantis)* has the potential to reduce HA-BSI, morbidity as well as mortality.

Conclusion

As medical interventions advance, and extremely preterm neonates survive in greater numbers, the incidence of HA-BSI increases. Probiotics could play an important role in preserving gut integrity and preventing severe infections in preterm neonates. In this RCT, a multi-strain probiotic containing *Lactobacillus acidophilus*, *Bifidobacterium bifidum* and *Bifidobacterium infantis* given daily to preterm neonates significantly reduced the incidence of HA-BSI.

Ethics

Ethics Committee Approval: Ethical approval was granted by the Health Research Ethics Committee of the Faculty of Health Sciences of Stellenbosch University as well as Tygerberg Hospital (S20/07/178).

Informed Consent: Written informed consent was obtained from each neonate's mother.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: M.S., M.M.V.W., A.N.H.B., A.D., E.V.N., Design: M.S., M.M.V.W., A.N.H.B., A.D., E.V.N., Analysis or Interpretation: C.L., Literature Search: M.S., M.M.V.W., A.N.H.B., A.D., E.V.N., Writing: M.S., M.M.V.W., A.N.H.B., A.D., C.L., E.V.N.

Conflict of Interest: The authors declared that there were no conflicts of interest.

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Original Article

Comparison of Invasive Measurement and Two Non-Invasive Measurements in the Diagnosis of Neonatal Hyperbilirubinemia

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ABSTRACT

Aim: Reliable non-invasive methods are required for the diagnosis of indirect hyperbilirubinemia (IHB) in infants. We compared the measured total serum bilirubin (TSB) levels against the transcutaneous and BiliCam methods.

Materials and Methods: This analytical study was performed in a neonatal intensive care unit of a hospital in Turkey. We included 70 infants whose families gave voluntary and written consent, including those infants with a low, medium, and high risk of hyperbilirubinemia, birth weight >1,500 g, and late preterm infants. We measured the TSB and compared it with bilirubin levels obtained via the transcutaneous and BiliCam measurement methods. The relationships between the data were determined using descriptive statistical methods; continuous data showing normal distribution were analyzed using Pearson correlation coefficient, and data that were not normally distributed were analyzed using Spearman correlation analysis.

Results: A statistically significant and positive correlation was observed between the levels of TSB and transcutaneous bilirubin before phototherapy (PT), whereas a moderate relationship was observed in these values after PT ($p<0.01$). A significant positive and moderate relationship was observed between the TSB levels and bilirubin levels measured using BiliCam before PT ($p<0.01$), and a weak relationship was observed between these values after PT ($p<0.05$).

Conclusion: Our results show that considering measurement of TSB as a reference method, the transcutaneous and BiliCam methods can be used as screening methods to detect IHB.

Keywords: Hyperbilirubinemia, transcutaneous, infant, smartphone

Introduction

Clinically, hyperbilirubinemia is observed in at least two-third of infants during their first week of life (1).

Risk factors for hyperbilirubinemia include uridine diphosphate-glucuronyl transferase 1A1 polymorphism in breast-fed infants (2), vitamin D deficiency (3), birth weight <2,500 g, pathological weight loss, exclusive breastfeeding (4), glucose 6 phosphate dehydrogenase (G6PD) deficiency (5), ABO and Rh incompatibility (6), late preterm babies (>40 weeks) (7), sibling history of jaundice, cephalohematoma (8), and some drugs used in pregnancy (9).

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Previous studies have reported the various complications of hyperbilirubinemia. A previous study showed a relationship between neonatal total serum bilirubin (TSB) levels and asthma diagnosis before the age of 7 years (10). Cochlear and auditory functions may be affected in babies with indirect hyperbilirubinemia (IHB) (bilirubin levels >20 mg/dL) for a long time (11). Infants with hyperbilirubinemia at birth are at higher risk of being diagnosed with sensorineural hearing loss (12). Hyperbilirubinemia may be associated with neurodevelopmental disorders (13). Kernicterus continues to be reported to date, particularly in developing countries, and this is a public health problem (14). Cases of kernicterus have also been reported Turkey (15,16). Thus, the diagnosis and treatment of hyperbilirubinemia is necessary.

Measurement of TSB levels is one of the methods used in the diagnosis of IHB. Central laboratories typically consider measurement of TSB as the gold standard for the detection of IHB, and this method is used to evaluate the efficiency of other bilirubin measurement methods. However, determination of TSB levels requires collection of venous blood, which is an invasive method and is painful for the infants and may not always guarantee sufficient blood supply (1). The environment in the neonatal intensive care unit is stressful for babies. Separation from the mother and exposure to recurrent pain are the main factors causing stress in these babies. If the recurrent pain in these is left untreated, these infants may experience permanent neurological and behavioral problems in the future and it may impair their pain perceptions and neuroendocrine stress responses. Pain awareness, approach to pain, and pain control and treatment are extremely important in infants. The most effective approach for controlling pain is to reduce painful interventions (17). In addition, early discharge without appropriate follow-up, lack of knowledge of the mother, cultural practices, and the use of traditional treatments may limit or delay the detection of jaundice and its subsequent treatment (18). Therefore, reliable non-invasive interventions are needed to detect hyperbilirubinemia.

The results of a study by Akman et al. (19) showed a significant correlation between the non-invasive method for determining transcutaneous bilirubin (TcB) and TSB measurement; a weak correlation was observed in cases of TSB levels >15 mg/dL, but a significant correlation was observed when TSB levels were <15 mg/dL. Furthermore, measurements of TcB levels can be performed reliably, quickly, and easily in infants for the screening of IHB, and babies at a low risk of IHB can also be detected using this method. Additionally, this method prevents unnecessary blood collection from infants (20). Phototherapy (PT) significantly affects the accuracy of transcutaneous bilirubinometry. TSB evaluation is required when considering the treatment of hyperbilirubinemia via TcB measurement (21).

One of the non-invasive methods for the determination of bilirubin levels is the BiliCam-estimated bilirubin (BCB) method. Taylor et al. (22) compared the BiliCam method, which is a new method for detecting IHB using an application downloaded onto a smartphone and a color calibration card placed on the sternum of the baby, with the method for measuring TSB levels. The results of their study showed that BCB had sufficient accuracy and could be used as a screening method to detect IHB. The results of a previous study showed that hyperbilirubinemia could be detected successfully and rapidly using the smartphone application, and the success rate of detection using this method was 85% (23).

To date, no studies have compared the method for determination of TSB with TcB and the BiliCam method. Comparisons of serum bilirubin concentrations measured using the TSB measurement with those measured using the TcB and BCB methods are required to determine which method is more advantageous and in which situation so that the most appropriate method of measurement may be used according to the specific situations. The diagnosis of IHB in infants is crucial; moreover, it is necessary in order to reduce the effects of IHB complications and invasive interventions on the infant. Therefore, in this study, we compared the method for determining TSB levels, as a reference method, with the TcB and BCB methods for the detection of neonatal hyperbilirubinemia.

Materials and Methods

Setting

This analytical study was conducted in a neonatal intensive care unit of a hospital in Turkey.

Participants

The study sample consisted of late preterm and term infants with low, moderate, and high risk of hyperbilirubinemia in a neonatal intensive care unit, diagnosed with IHB, having a birth weight >1,500 g, and requiring PT. Infants whose families volunteered and gave written consent were included in this study. We included healthy infants at a low risk delivered at 38 weeks, infants

at intermediate risk delivered at 38 weeks with the presence of risk factors, healthy infants delivered at 35-37 weeks 7 days, and infants at high risk delivered at 35-37 weeks 7 days with the presence of risk factors. Risk factors included iso-immune hemolytic disease, G6PD deficiency, asphyxia, severe lethargy, heat instability, sepsis, acidosis, and/or serum albumin levels <3 g/dL (1).

On the basis of the power analysis made according to the sensitivity and selectivity values obtained from a reference study (22), 70 infants were included in this study to obtain 80% power at 95% confidence level in the power analysis calculated with an 85% sensitivity value and a 65% selectivity value.

Data Collection

The descriptive information of the infants was recorded via the "neonatal descriptive information form," and the results of measurement of bilirubin levels were recorded with the "measurement results registration form". Bilirubin concentrations were measured using the TcB and BCB methods after blood was collected from the infants for the measurement by TSB; the bilirubin concentrations were measured before and after the commencement of PT. Data were collected between January, 2020 and September, 2020.

Measurements Tools

The neonatal descriptive information form was used in this study. This form includes the sociodemographic characteristics of the infants and those features relating to hyperbilirubinemia. It consists of the following questions: Birth weight, current weight, method of delivery, method of feeding, history of jaundice in siblings, blood type of the baby, maternal blood type, and the presence of risk factors for IHB.

The measurement results registration form. This form includes the results of the bilirubin levels measured using the TSB, TcB, and BCB methods before and after PT.

Measurements using a TcB meter. Before the initiation of PT, the bilirubin levels were measured by placing a TcB meter on the sternum of the baby. When measuring with TcB, the area of the sternum was preferred for hygiene reasons and ease of measurement. After the initiation of PT, the measurements were made by placing the bilirubin meter on the covered hip bone, which was not exposed to PT.

Measurements using a smartphone application and color calibration cards (Figure 1). This application, which is freely available online, was downloaded onto an iPhone 5s. This application was used together with color calibration cards measuring 5×5 cm which had hollow squares with a variety of colors. The color calibration card was downloaded onto the phone using the application and a printout was taken.

When measuring with BCB before PT started, the measurements were made on the sternum of the infant. After the PT had started, the measurements were made over the covered hip bone which was not exposed to PT. Before PT, the sternum area was preferred for hygiene and ease of measurement. Since the sternum was affected by PT, after PT, measurements were made on the covered hip bone. After opening the application on the smartphone, the application is started by placing a color calibration card on the sternum/hip bone of the infant. A red square appears on the smartphone screen. When this red square is matched correctly with the color calibration card and when the lighting is sufficient, it will turn into a green square. The application automatically shows the bilirubin value by capturing both flash and non-flash images using the smartphone camera.

It is recommended that the smartphone be disinfected using 70% isopropanol before and after each measurement to avoid any risk of contamination. Accordingly, we disinfected the smartphone and the TcB meter using 70% isopropanol before and after each measurement. The color calibration card was changed after every measurement.

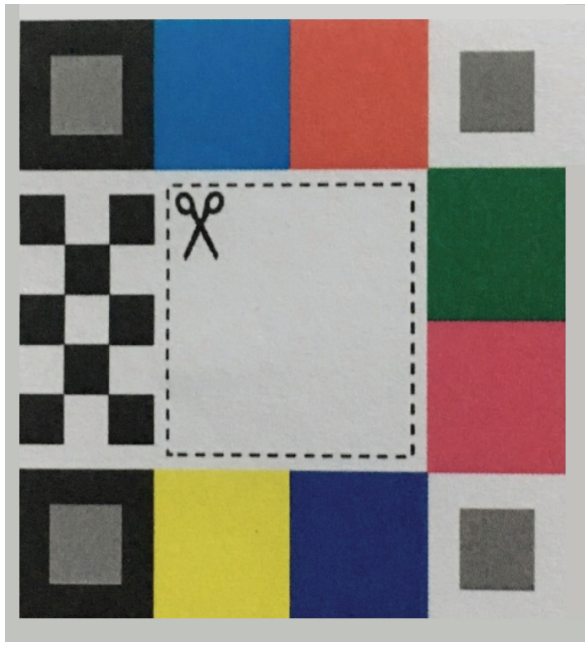

Figure 1. Design of the color calibration card used in our studies

Ethical Considerations

Permission to conduct this study was obtained from the Pamukkale University Non-Interventional Clinical Research Ethics Committee (27.07.2018-E.50623) and the hospital where the research was performed. Parents of the infants included in the sample were informed about this study and their written consent was obtained.

Statistical Analysis

The data obtained from this study were analyzed using the Statistical Package for the Social Sciences (SPSS) 25 package program. Continuous variables were represented as mean ± standard deviation and categorical variables as numbers and percentages. The conformity of the data to normal distribution was examined using the Kolmogorov-Smirnov test. Continuous data showing normal distribution were analyzed using Pearson correlation coefficient, and data that were not normally distributed were analyzed using Spearman's correlation coefficient. Statistical significance was evaluated as $p<0.01$ and $p<0.05$.

Results

The findings regarding the sociodemographic characteristics of the infants included in this study are given in Table I. The average age of the infants was 4.15±1.93 days (range, 1-7 days) and 54.3% of the infants were male.

The birth weight of the infants was 3,161.57±514.14 g and their average weight at the time of this study was 3,007.14±497.56 g. Results regarding the method of delivery showed that 55.7% of the infants were born by normal delivery, and 94.3% of infants had no history of jaundice in their siblings. Determination of the blood groups of the baby showed that 40% of the infants belonged to the A blood group and 90% were Rh positive. Among the mothers, 51.4% belonged to the O blood group, and 12.9% of them were Rh negative. ABO incompatibility was reported in 31.4% of the infants, Rh incompatibility in 14.3% of the infants, and 95.7% of them did not have any risk factors for IHB (Table II).

Table I. Distribution of the sociodemographic characteristics of the infants

	Mean	Standard deviation
Age (days)		
(Minimum-maximum: 1-7 days)	4.15	1.93
	Frequency	**%**
Gender		
Male	38	54.3
Female	32	45.7

Table II. Distribution of the characteristics of infants with risk of hyperbilirubinemia

	Mean	Standard deviation
Birth weight (g)		
(Minimum-maximum: 2,300-4,450)	3,161.57	514.14
Current weight (g)		
(Minimum-maximum: 2,100-4,000)	3,007.14	497.56
	Frequency	**%**
Method of delivery		
Normal	39	55.7
Caesarean	31	44.3
Method of feeding		
Oral (breast milk and formula)	48	68.6
Oral (breast milk)	10	14.3
Oral and intravenous	7	10.0
Intravenous	5	7.1
History of jaundice in sibling		
No	66	94.3
Yes	4	5.7
Baby blood groups		
A	28	40.0
O	24	34.3
B	11	15.7
AB	7	10.0
Rh positivity of the baby		
Positive	63	90.0
Negative	7	10.0
Mother blood groups		
O	36	51.4
	Frequency	**%**
A	20	28.6
AB	10	14.3
B	4	5.7
Rh positivity of the mother		
Positive	61	87.1
Negative	9	12.9
ABO incompatibility		
No	48	68.6
Yes	22	31.4
Rh incompatibility		
No	60	85.7
Yes	10	14.3
Risk factors		
No	67	95.7
Yes	3	4.3

The distribution of bilirubin levels in the infants measured using the TSB, TcB, and BCB methods before and after PT are shown in Table III.

The mean values of TSB, TcB, and BCB before PT were 17.35±4.86 mg/dL, 15.37±3.75 mg/dL, and 14.14±1.75 mg/dL, respectively, whereas these values after PT were 9.16±3.21 mg/dL, 7.35±2.75 mg/dL, and 8.57±2.53 mg/dL, respectively (Table III).

Before PT, a statistically significant positive and strong relationship was observed between the levels of TSBs and TcB values ($p<0.01$). After PT, a statistically significant positive and moderate relationship was determined between the levels of TSB and TcB ($p<0.01$, Table IV).

Before PT, a statistically significant positive and moderate relationship was observed between the levels of TSB and BCB ($p<0.01$). A statistically significant positive and weak relationship was observed between the levels of TSB and BCB after PT ($p<0.05$, Table V).

Table III. Distribution of bilirubin values before and after phototherapy of infants

	Before PT mean ± SD	Lower-Upper	After PT mean ± SD	Lower-Upper
TSB (mg/dL)	17.35±4.86	5.94-33	9.16±3.21	4.19-22.68
TcB (mg/dL)	15.37±3.75	6.4-22	7.35±2.75	1.7-14.2
BCB (mg/dL)	14.14±1.75	9.6-16.9	8.57±2.53	3.7-14.1

PT: Phototherapy, TSB: Total serum bilirubin, TcB: Transcutaneous bilirubin, BCB: BiliCam-estimated bilirubin, SD: Standard deviation

Table IV. Comparison of the relationship between total serum bilirubin levels and bilirubin levels measured using transcutaneous bilirubin measurement in infants before and after phototherapy

	R	p
Before phototherapy	0.765**	0.000
After phototherapy	0.610**	0.000

**The correlation is significant at the 0.01 level

Table V. Comparison of the relationship between total serum bilirubin levels and bilirubin levels measured using BiliCam in infants before and after phototherapy

	R	p
Before phototherapy	0.572**	0.000
After phototherapy	0.283*	0.017

*The correlation is significant at the 0.05 level
**The correlation is significant at the 0.01 level

Discussion

Our results showed that 51.4% of the women had blood group O, and 12.9% of the mothers were Rh (-) (Table II). ABO incompatibility was reported in 31.4% of the infants and 14.3% of them had Rh incompatibility (Table II). ABO and Rh incompatibility is one of the risk factors for IHB (6). Our findings were similar to those reported in previous studies.

The maximum TSB, TcB, and BCB values before PT were 33 mg/dL, 22 mg/dL, and 16.9 mg/dL, respectively. The maximum TSB, TcB, and BCB values after PT were 22.68 mg/dL, 14.2 mg/dL, and 14.1 mg/dL, respectively (Table III).

Taylor et al. (22) determined that a cut-off value of 13 mg/dL for BCB. Ercan and Özgün (24) recommend using a TcB cut-off value of 222 µmol/L (12.98 mg/dL). Chokemungmeepisarn et al. (25) recommended using a cut-off value of + 3 mg/dL for TcB. Hulzebos et al. (26) reported the cut-off value of TcB as + 50 µmol/L (2.92 mg/dL). These studies suggest an approximate cut-off value of 13 mg/dL when using these two non-invasive measures. Since our study was conducted without using a cut-off value, high bilirubin values negatively affected our study results.

Thus, our results show a strong relationship between TSB and TcB measurement values before PT, whereas a moderate relationship was observed in these values after PT (Table IV).

Castro et al. (27) showed a significant positive correlation between TSB and TcB in regions not exposed to PT. However, since the reliability of these results was not established, they do not recommended using these results as a guide for clinical decisions regarding the duration of PT (28).

Previous studies examining the reliability of bilirubin measurement methods showed that the measurement of TcB on a skin patch after PT was found to be reliable (28-30).

Hulzebos et al. (26) measured bilirubin levels over the covered hip bone in premature babies with a gestational age of ≤32 weeks. The results of the study by Hulzebos et al. (26) showed that the requirement of a 40% reduction in TSB levels was achieved by using TcB + 50 µmol/L (2.92 mg/dL) as the cut-off level.

We measured bilirubin levels with TcB after PT over the covered hip bone, that is, from under the diaper. The results obtained may not be consistent because of the difficulty in completely protecting the measurement area from PT light because of the constant movement of the baby.

Our results showed a moderate correlation between TSB measurement values before PT and BCB measurement

values. After PT, a weak correlation was found (Table V). Taylor et al. (22) reported that a cut-off of 13 mg/dL could be used for BCB before PT. To date, no study has investigated the effect of PT on BCB measurement.

In our study, the bilirubin measurements with BCB were made over the covered hip bone, that is, under the diaper, after PT. This result is thought to be affected by the difficulty of fully protecting the measurement area from PT light due to the baby being mobile, and the difficulty in fixing the color calibration card due to the area where the measurement was made.

Study Limitations

Limitations of this research; those with a gestational age of ≤35 weeks and an age of >7 days and those who had received PT treatment previously were not included in this study. Non-invasive measurements were made from the sternum before PT and over the covered hip bone after PT. No cut-off value was used in TSB values.

Conclusion

Comparison of TSB with TcB and BCB is necessary to determine which method is more advantageous and in which situation to use the most appropriate measurement method for the detection of IHB in order to decrease the effects of IHB complications and limit invasive procedures on the infant. We compared TSB as a reference method with TcB and BCB for the detection of neonatal hyperbilirubinemia. Our results show that TcB and BCB measurement methods can be used for the detection of IHB, considering TSB as a reference method. The TcB and BCB measurement methods can be used to detect bilirubin in the clinic, but if a change in treatment is considered, it should be confirmed by using TSB levels. As our study was conducted without using cut-off values, it may not be reliable for high bilirubin values. In terms of cost and accessibility of the device, the use of a transcutaneous device in neonatal intensive care units may reduce the need for blood sampling for TSB. For TcB and BCB measurements after PT, protecting the measurement area from PT light can increase the reliability of both BCB and TcB measurement values.

Further studies should be performed in order to determine the accessibility and ease of use of the BiliCam method by the parents. Active use of the BCB method can detect IHB.

Ethics

Ethics Committee Approval: Permission to conduct this study was obtained from the Pamukkale University Non-Interventional Clinical Research Ethics Committee (27.07.2018-E.50623) and the hospital where the research was performed.

Informed Consent: Parents of the infants included in the sample were informed about this study and their written consent was obtained.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Z.K.B., B.Ç., Desing: Z.K.B., B.Ç., Data Collection or Processing: Z.K.B., B.Ç., Analysis or Interpretation: Z.K.B., B.Ç., Literature Search: Z.K.B., B.Ç., Writing: Z.K.B., B.Ç.

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Original Article

Micafungin Effectiveness in Treating Pediatric Patients with Proven Candidemia

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ABSTRACT

Aim: Micafungin is one of three currently available echinocandin for the treatment of candidiasis and candidemia. We aimed to discuss the effectiveness of micafungin and any possible side effects in the treatment of proven candidemia in children.

Materials and Methods: In this study, children who were treated with micafungin for proven candidemia between May, 2017 and October, 2019 were included. The time to achieve negative culture, liver and renal functions as well as blood counts were recorded using the hospital data system.

Results: Forty-five patients (52.3%) who received micafungin for proven candidemia were included in this study. The median age of the children who received micafungin due to invasive candidiasis (IC) was 4 months (range: 12 days to 216 months). Of these 45 IC patients, 10 (22.2%) were neonates, 19 (42.2%) were infants, 11 (24.4%) were between 1 and 5 years old, and 5 (11.1%) were between 10-18 years old. The median duration of micafungin treatment to culture negativity for *C. albicans* related candidemia episodes was shorter (6 days, 1-26 days) than *non-albicans Candida* spp. related candidemia episodes (7 days, 1-35 days) (p=0.10). Culture negativity could not be achieved at the end of the 14th day of micafungin treatment in 15 of the 45 (33.3%) candidemia episodes. The most commonly isolated *Candida* spp. in patients with treatment failure was *C. parapsilosis* (n=6), followed by *C. albicans* (n=5), *C. guilliermondii* (n=1), *C. tropicalis* (n=2) and *C. tropicalis* and *C. guilliermondii* co-infection (n=1) respectively. None of the patients developed side effects due to micafungin treatment.

Conclusion: Micafungin was found to be safe and effective for the treatment of culture proven candidemia in pediatric patients, including neonates.

Keywords: Micafungin, effectiveness, safety, candidemia, antifungal resistance, pediatric patients

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Introduction

Invasive fungal infections (IFIs) caused by *Candida* spp. are important causes of morbidity and mortality in both immunocompromised and hospitalized patients. Candidemia is one of the most common cause of pediatric health care-associated bloodstream infections in the world (1-4).

Due to the emergence of treatment resistance to broad spectrum triazole antifungals, new treatment options for IFIs are required. Echinocandins provide clinicians with an alternative treatment option as they are well tolerated, have rapid antifungal activity, favorable pharmacokinetics and some of them do not require a loading dose. Micafungin is an echinocandin with demonstrated effectiveness for the treatment of invasive candidiasis (IC) and for the prophylaxis of *Candida* infection in patients undergoing allogeneic hematopoietic stem cell transplantation, or in those who are expected to have neutropenia for ≥10 days (5,6).

Micafungin is a non-competitive, concentration-dependent inhibitor of the enzyme 1,3-b-D-glucan synthase and, consequently, inhibits the synthesis of 1,3-b-D-glucan (an integral component of the fungal cell wall, which is not present in mammalian cells). Micafungin was approved by the European Medicines Agency in 2008 for the treatment of IC, for the prophylaxis of *Candida* infection in patients undergoing allogeneic haematopoietic stem cell transplantation or for patients who are expected to have neutropenia (absolute neutrophil count <500 cells/μL) for 10 or more days for children (including neonates) and adolescents <16 years of age (7).

Micafungin is well-tolerated by pediatric patients. The incidence of treatment-related adverse events is lower in pediatric patients (8,9). The most frequently reported treatment-related adverse events are nausea (2.8% of subjects), elevated alkaline phosphatase (2.7%), phlebitis (2.5%), vomiting (2.5%), elevated aspartate aminotransferase (AST) levels (2.3%), hypokalemia (2.1%), fever (2.1%), and elevated alanine aminotransferase (ALT) levels (2%) (9). However, there are limited data available regarding the effectiveness and safety of micafungin in children (10-14).

The aim of the current study was to evaluate the effectiveness and tolerability of intravenously administered micafungin for proven IC in pediatric patients.

Materials and Methods

Patient Selection and Study Design

All children, including neonates, who were hospitalized in the pediatric wards of University of Health Sciences Turkey, Dr. Behçet Uz Pediatric Diseases and Surgery Training and Research Hospital, and who had received micafungin due to proven IC between May, 2017 and October, 2019 were included in this retrospective study. The patients' risk factors, underlying diseases, type of unit of hospitalization, routine laboratory assessments of biochemistry (serum concentrations of liver enzymes, bilirubin, creatinine, urea, albumin, electrolytes) and blood counts as well as the daily doses and durations of micafungin treatment, concomitant antibiotics, clinical responses and adverse effects were obtained from their electronic medical records. Biochemical parameters such as serum concentrations of liver enzymes, bilirubin, creatinine, urea, albumin, and electrolytes were recorded in order to evaluate the safety of micafungin.

Micafungin doses of 4-10 mg/kg in neonates and 2-4 mg/kg in children with IC were used (15). The efficacy end-point was defined as being alive and fungal free [based on improvement of clinical symptoms and laboratory findings (culture)].

Case Definitions

Candidemia is defined as the presence of the growth of any *Candida* species in at least one blood culture obtained by either peripheral venipuncture or through an indwelling central venous catheter (CVC). When the same isolate is detected in a peripheral blood culture and catheter-drawn blood culture obtained at least 2 hours apart, candidaemia is considered as a CVC related bloodstream infection. Time to mycological eradication represents the number of days from the initiation of treatment to the first day of blood culture negativity for *Candida* species. Treatment failure was defined as death within 14 days of the initiation of therapy or ≥1 positive blood culture for *Candida* spp. 14 days or more after the initiation of antifungal therapy. If a patient died due to a different identifiable cause, this death was not seen as treatment failure.

Death which ensues within 30 days of the onset of candidemia with no apparent alternative cause is recognized as a candidemia-attributable mortality.

For the assessment of any potential side effects, the following standard values were applied: AST, normal range 25-85 U/L; alanine ALT, normal range 12-93 U/L.

Statistical Analysis

All statistical analyses were performed using the SPSS package program for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA). Continuous data are expressed as mean±standard deviation and categorical variables are reported as percentages. The distribution of continuous variables was assessed by the Kolmogorov-Smirnov test. Probable associations among categorical variables were evaluated by the chi-squared test or Fisher's exact test. Fisher's exact test was applied if more than 20% of the categories have expected frequencies less than 5. Parametric and non-parametric continuous variables were analyzed by the independent t-test and the Mann-Whitney u test, respectively.

The inferential statistical analysis between the baseline values as well as the maximum and minimum parameters and the parameters at the cessation of antifungal treatment were carried out by Wilcoxon matched-pairs and were analyzed by the Friedman two-way analysis of variance by ranks, due to non-normally distributed values in at least one group or day, making a parametric analysis of variance (ANOVA) for repeated measures not appropriate. The analyses of the hepatic and kidney parameters are presented as median (minimum-maximum). P-values of ≤0.05 were defined as significant.

This study was approved by the Ethics Committee of University of Health Sciences Turkey, Dr. Behçet Uz Pediatric Diseases and Surgery Training and Research Hospital (date: 10.12.2019, approval no: 13399118-799).

Results

In this study, 86 children in total with a median age of 3 months (9 days-17 years), had received micafungin. Fifty-eight (67.4%) of the patients were male (Female/Male ratio=0.48). Forty-five patients (52.3%) who had received micafungin for proven candidemia were included in this study. The median age of the children who received micafungin due to IC was 4 months (range=12 days-216 months). Of these 45 IC patients, 10 (22.2%) were neonates, 19 (42.2%) were infants, 11 (24.4%) were between 1-5 years old, and 5 (11.1%) were between 10-18 years old. Four of the 10 (40%) neonates were premature. Ten (22.2%) patients had been hospitalized in the neonatal intensive care unit, 16 (35.6%) patients in the pediatric intensive care unit, 9 (20%) patients in the surgical intensive care unit, 5 (11.1%) patients in the hematology-oncology unit, 1 (2.2%) in the pediatric infectious diseases ward and the other 4 (8.8%) patients in general pediatric wards at the time of micafungin treatment. There were 27 children with underlying diseases including a history of intra-abdominal surgery (n=13), congenital heart disease (n=3), hemato-oncological disease (n=5), and immune deficiency (n=6). The baseline characteristics of these patients are presented in Table I.

Twenty-five (55.6%) IC patients had been receiving fluconazole treatment before switching to micafungin treatment. Twenty-three (51.1%) *Candida* spp. cases were resistant to fluconazole, which was the reason for switching to micafungin treatment. Fourteen (31.1%) *Candida spp.* were *C. albicans*, and the thirty-one (68.9%) were *non-albicans Candida* spp. The most commonly isolated *Candida* spp. was *Candida parapsilosis (C. parapsilosis)* (n=15) followed by *C. albicans* (n=14), *C. tropicalis* (n=5), *C. glabrata* (n=4), *C. guilliermondii* (n=3), *C. krusei* (n=2), and *C. pelliculosa* (n=1). Additionally, *C. guilliermondii* and *C. tropicalis* were isolated concomitantly in 1 patient (Figure 1).

The median duration of micafungin treatment in the 45 IC cases was 14 days (3-36 days). The median duration of

Table I. The demographic and clinical characteristics of the patients

Age**	4 months (range: 12 days-216 months).
Gender (male/female)*	31/14 (68.9 vs. 31.1, F/M: 0.45)
Prior fluconazol treatment*	25 (55.6)
Neonatal intensive care unit (n, %)*	10 (22.2)
Term*	6 (60)
Preterm*	4 (40)
Gestational week (weeks)	35 (min.: 26/max.: 40)
Gender (male/female)*	8/2 (M/F: 4)
Hospital ward*	
Neonatology	10 (22.2)
Pediatric intensive care unit	16 (35.6)
Surgical intensive care unit	9 (20)
Oncohematology	5 (11.1)
Others	5 (11.1)
Underlying disease***	
Intra-abdominal surgery	13
Congenital heart disease	3
Hematological malignancy	5
Primary immune deficiency	6
*: n (%) **: Median (minimum-maximum)	***= n

micafungin treatment to culture negativity for candidemia episodes was 6.5 days (1-35 days). Culture negativity could not be achieved in 2 patients. Two patients with IC [*C. parapsilosis* (n=1) and *C. albicans* (n=1)] died, one due to IC and the other due to concomitant gram negative bacterial sepsis. The median duration of micafungin treatment to culture negativity *for C. albicans* related candidemia episodes was shorter (6 days, range: 1-26 days) than *non-albicans Candida* spp. related candidemia episodes (7 days, range: 1-35 days) (p=0.10). Culture negativity could not be achieved at the end of the 14th day of micafungin treatment in 15 (33.3%) of the 45 IC episodes. The most commonly isolated *Candida* spp. in those patients with treatment failure was *C. parapsilosis* (n=6), followed by *C. albicans* (n=5), *C. guilliermondii* (n=1), *C. tropicalis* (n=2) and *C. tropicalis* and *C. guilliermondii* coinfection (n=1). One patient

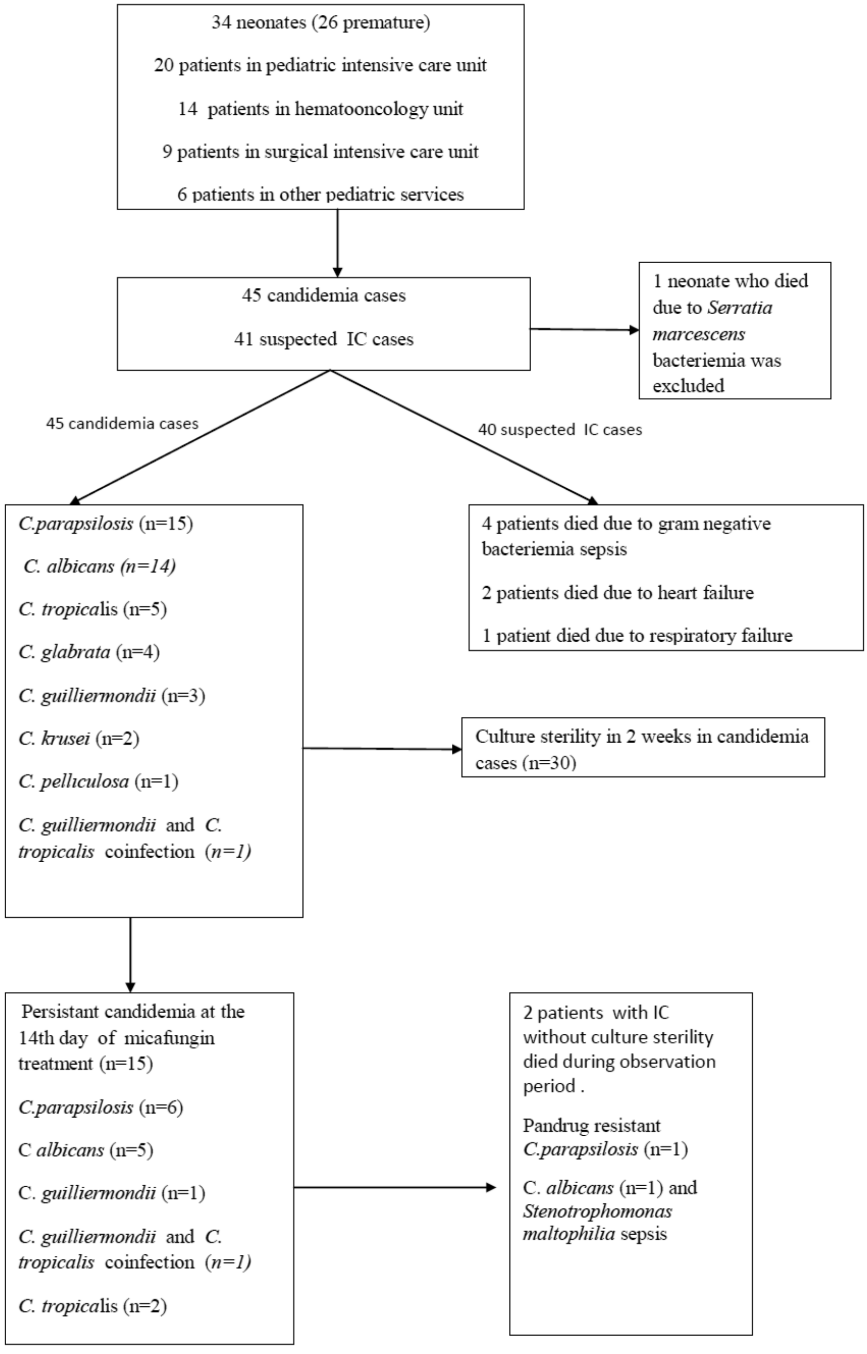

Figure 1. Outcomes of patients who received micafungin

with pan-drug resistant *C. parapsilosis* candidemia died during the observation period and this was attributed to treatment failure.

Non-albicans Candida spps. were more commonly isolated from patients with treatment failure at the 14th day of micafungin treatment (n=10, 66%).

The median serum thrombocyte value was statistically significantly higher at the end of micafungin treatment compared to the median value before treatment (p<0.001). Median thrombocyte values taken prior to micafungin treatment and at the end of micafungin treatment were 145x10^3/µL (15-772x10^3/µL) versus 272x10^3/µL (19-937x10^3/µL) respectively (Table II).

Safety

None of the patients had treatment interruption because of adverse drug reactions. Serum AST and ALT levels were higher in those IC patients with prior fluconazole treatment (Table II). Serum alanine aminotransferase levels were statistically significantly higher in the group who had received fluconazole treatment before switching to micafungin treatment (21.7%, p=0.005). Serum ALT levels in the group who had received fluconazole treatment before switching to micafungin treatment were statistically significantly decreased after switching to micafungin treatment (p=0.05).

Table II. Laboratory change before and after micafungin treatment

	Before micafungin treatment	After micafungin treatment	p-value
Serum AST (IU/L)	37 (8-885)	39 (7-685)	0.61
Serum ALT (IU/L)	27 (6-790)	27 (6-174)	0.11
Serum creatinine (mg/dL)	0.5 (0.3-8)	0.3 (0.5-5)	0.86
Serum sodium (mmol/L)	137 (123-147)	137 (130-164)	0.40
Serum potassium (mmol/L)	4.3 (2.6-5.7)	4.5 (2.7-5.1)	0.80
Hemoglobin (gr/dL)	9.7 (6.2-16.9)	9.7 (7-14.5)	0.68
Leucocyte (x10^3/µL)	9.2 (0.37-35.6)	10.6 (1.6-48.9)	0.13
Absolute neutrophil count (x10^3/µL)	4.2 (0.18-24.7)	4.1 (0.15-33.7)	0.74
Thrombocyte (x10^3/µL)	**129 (15-772)**	**250 (13-937)**	**<0.001**

Values are given as median (min.-max.)
AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, min.: Minimum, max.: Maximum

Discussion

In this study, our experience with intravenous micafungin treatment in 45 pediatric patients with proven IC were reviewed. *Non-albicans Candida* spp. have been more commonly isolated than *C. albicans* isolates in pediatric studies from our country (16-19). Fifteen (17.4%) patients died during the observation period but only 1 patient's death was attributable to candidemia due to *C. parapsilosis* candidemia.

Fourteen *Candida* spps. were resistant to all antifungals, minimum inhibitory concentration (MIC) for micafungin was the lowest which was the reason for the treatment choice. The increase in antifungal resistance of *Candida* spp. was thought to be the reason for persistent candidemia on the 14th day of micafungin treatment. However, culture negativity was achieved in 12 pan-drug resistant IC episodes after the 14th day of micafungin treatment. Two patients (one patient with a diagnosis of cerebral palsy and another with tetralogy of Fallot) died during candidemia episodes and culture negativity could not be achieved within 2 weeks in these 2 patients. *Candida parapsilosis* cultured in the blood culture of 1 of these 2 patients was resistant to all antifungals. In the other patient who was operated due to tetralogy of Fallot, *C. albicans* was the reason of candidemia but death was due to Gram-negative bacterial sepsis.

Micafungin treatment in neonates, including premature newborns, has not been extensively studied to date. In our study, treatment related side effects and treatment failure were not seen in neonates. In our study, all of the neonates were effectively treated with micafungin and all were culture-negative within 2 weeks. Benjamin and colleagues compared the efficacy, safety and pharmacokinetics of intravenous micafungin with intravenous amphotericin B deoxycholate in a phase 3, randomized, double-blind, multicenter, parallel-group, non-inferiority trial performed on infants between 2 and 120 days of age with proven IC. A total of 20 infants received micafungin, and 10 received amphotericin B deoxycholate. Although their study was terminated early due to low recruitment, fungal-free survival was observed in 12 out of the 20 [60%; 95% confidence interval (CI): 36-81%] infants treated with micafungin versus 7 of the 10 (70%; 95% CI: 35-93%) infants treated with amphotericin B deoxycholate (20).

Micafungin treatment in pediatric hematological malignancy is limited. In our study, 5 patients with hemato-oncological malignancy received micafungin and all of these patients were effectively treated. These results are similar to a study conducted in neutropenic patients. The

authors concluded that micafungin is effective against IC/candidaemia in those patients with neutropenia, irrespective of neutropenia duration or cultured *Candida* spp. (21).

In a study conducted on 8 pediatric patients using micafungin (≥3 doses) who had breakthrough candidemia (BC), the causative strains of BC were *C. parapsilosis* in seven of these patients. The authors concluded that immunocompromised patients may develop BC caused by micafungin-susceptible strains (21). In our study, persistent candidemia at the 14th day of micafungin treatment was seen in 15 cases with *Candida* spp., but culture negativity was achieved in 13 of these IC episodes.

In one study, micafungin was commenced for 174 courses in 148 patients, including 135 adults and 13 children aged under 18 years (10 of whom were under paediatric oncology care, 2 of whom were neonates and 1 was in general pediatric care). The authors concluded that micafungin was clinically effective for the treatment of IC and Aspergillus infections, and in line with our study results that micafungin usage did not increase the risk of liver dysfunction (12).

The development of the azole antifungals has enhanced treatment options for fungal infections and their reduced host toxicity has led to their widespread use. In our study, azole resistance of *C. albicans* and *non-albicans Candida* spp. were 50% and 61.9%, respectively. Consequently, with their extensive use, it is perhaps not surprising that resistance to these agents, particularly fluconazole, is encountered (22,23).

Echinocandins are fungicidal and have increased activity *in vitro* compared to amphotericin B deoxycholate and azoles against biofilms formed by *Candida* spp. Therefore, the most recent guidelines of the European Society of Clinical Microbiology and Infectious Diseases for the prevention and management of invasive infections in neonates and children support the increasing use of echinocandins in pediatric patients (24). In one study, conducted in 110 pediatric patients published in 2019, the authors concluded that micafungin was effective and well-tolerated as a prophylaxis against IFIs in pediatric onco-hematology patients and for curative purposes in pediatric and neonatal ICU patients, similar to our results (13).

Study Limitations

There are some limitations present in our study. As with any study with the sample size used in this study, the generalizability of our findings is limited. Additionally, this was a retrospective study, which has inherent limitations when compared to randomized clinical trials. Also, this study included all children, including neonates with different underlying diseases, co-morbidities and risk factors which might have caused bias for the outcome.

Conclusion

As a conclusion, micafungin was curative, especially in neonates, when used to treat IC. Pan-drug resistant candidemia was the reason of death for one patient included in our study. More aggressive treatment options should be chosen to treat pan-drug resistant IC cases. Additionally, in those centers with reports of emerging fluconazole resistant *Candida* spp. and *non-albicans Candida* spp., micafungin is a reliable and effective choice for empirical treatment for suspected candida infections of children.

Ethics

Ethics Committee Approval: This study was approved by the Ethics Committee of University of Health Sciences Turkey, Dr. Behçet Uz Pediatric Diseases and Surgery Training and Research Hospital (date: 10.12.2019, approval no: 13399118-799).

Informed Consent: Retrospective study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: K.Ö.A., Design: K.Ö.A., Data Collection or Processing: K.Ö.A., Analysis or Interpretation: K.Ö.A., Ş.Ç., H.A., N.B., İ.D., Literature Search: Writing: K.Ö.A., O.K., Ş.Ç., Ş.A., M.Ç., E.B., E.K., A.A.K., H.A., N.B., İ.D.

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Original Article

Assessment of Liver Dysfunction Using Combination Biomarkers in Children Living with HIV Infection

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ABSTRACT

Aim: Overall, around 14-18% of non-acquired immunodeficiency syndrome-related deaths are due to liver disease in human immunodeficiency virus (HIV) patients. With a prevalence of 15%, cirrhosis appears to be a more serious consequence. There are many non-invasive markers for assessing liver fibrosis but their utility in pediatric HIV patients has not been explored.

Materials and Methods: To assess the occurrence of liver dysfunction and the levels of combination biomarkers of liver dysfunction [aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio, AST-to-platelet ratio index (APRI), and fibrosis-4 (FIB-4) index] in HIV positive children. A total of 44 HIV positive children aged <15 years attending the antiretroviral therapy (ART) clinic were enrolled and evaluated for liver dysfunction using non-invasive biomarkers and ultrasonography (USG) scoring.

Results: Deranged biomarkers-AST/ALT ratios, APRI scores, and FIB-4 index were found in 95%, 6.8%, and 4.5% children respectively. 7% of children showed moderate to severe liver fibrosis on USG scoring. Also, anemia, nevirapine in ART regimen, longer ART duration, immunosuppression, and lower body mass index values were found as risk factors associated with deranged biomarkers.

Conclusion: Hepatic dysfunction is reflected by deranged AST/ALT ratios among HIV-positive children in this study. Further, the elevated APRI scores and FIB-4 index in some cases signal evolving liver fibrosis.

Keywords: HIV, hepatic dysfunction, liver fibrosis, biomarkers, APRI score, FIB-4 index

Introduction

Liver disease has emerged as the most common non-acquiredimmunodeficiency syndrome-related cause of death among human immunodeficiency virus (HIV)-positive patients, accounting for 14-18% of all deaths (1). Nearly half of deaths among hospitalized HIV-positive patients in the highly active antiretroviral therapy (ART) era have been attributed to liver diseases, which range from asymptomatic mild elevations of liver enzymes to cirrhosis and end-stage liver disease with all its complications (1). Liver cirrhosis is a more serious consequence and the prevalence of significant liver fibrosis in those with HIV approaches 15% (2). Patients with HIV have a proclivity to develop liver cirrhosis (2,3).

Liver biopsy is currently considered the gold standard for fibrosis assessment but carries many shortcomings (cost, invasiveness, and complications) (4). Recently, many non-invasive markers for assessing liver fibrosis have been developed for the assessment of liver fibrosis (studied in hepatitis B patients) (4). When liver disease is suspected, non-invasive screening methods such as the FibroScan may

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be beneficial but in many circumstances are prohibitively expensive and/or not accessible for children (5). Combination biomarkers, such as the aspartate aminotransferase (AST)/alanine aminotransferase (ALT) ratio, AST-to-platelet ratio index (APRI), and the fibrosis-4 (FIB-4) index have been reported as potentially useful for predicting hepatic fibrosis in children with non-alcoholic fatty liver disease (6), chronic viral hepatitis (7), or chronic liver disease from various etiologies.

There is a paucity of literature from India on liver dysfunction in children living with HIV. Also, the role of combination biomarkers in identifying liver disease in children with HIV is still not clear. In this paper, we have assessed the existence of liver dysfunction in Indian children living with HIV and also evaluated them for the presence of liver fibrosis using non-invasive markers of liver dysfunction.

Materials and Methods

This cross-sectional study was conducted at the ART clinic of the Department of Pediatrics at an institute located in New Delhi, India, after taking permission from the Institutional Ethics Committee. This study was conducted from March, 2018 to March, 2019 and a total of 44 HIV-positive children were enrolled and evaluated for liver dysfunction. The objective of this study was to assess the occurrence of liver dysfunction in children living with HIVand to assess the levels of combination biomarkers of liver dysfunction in these children. Children more than 18 months of age were confirmed to have HIV using three ELISA tests as per National AIDS Control Organization guidelines and those less than 18 months of age were diagnosed by virological tests [DNA Polymerase chain reaction (PCR)] from dried blood spots. All children less than 15 years of age with HIV were invited to be a part of this study. After taking written informed consent and assent (for children >7 years of age) from the parents or caregivers of the children, basic patient information such as their name, age, sex, demographic details, clinical history and examination, anthropometric measurements, immunological data, and details of antiretroviral treatment were recorded as per pre-structured pro-forma. All opportunistic infections were investigated and actively treated before ART commencement.

All HIV confirmed children attending the ART clinic, irrespective of their ART status, were enrolled and screened for liver dysfunction using non-invasive biomarkers of liver dysfunction. The liver function tests were evaluated using serum bilirubin, ALT, AST, and serum proteins levels. Abnormal liver enzymes aredefined as ALT or AST enzyme levels >1.25 times the upper limit of normal (ULN). The liver enzyme abnormalities were graded as follows; grade 1 hepatotoxicity: ALT or AST level 1.25 to 2.5 times ULN, grade 2 hepatotoxicity: ALT or AST level 2.6 to 5 times ULN, grade 3 hepatotoxicity: ALT or AST level 5.1 to 10 times ULN, grade 4 hepatotoxicity: ALT or AST level >10 times ULN (8,9). A routine hemogram including platelet counts and CD4 counts was obtained.

Also, any history of jaundice was recorded, and viral markers were taken in order to assess for the presence of any co-infection with hepatitis B or C.

Computation of biomarkers of liver dysfunction was carried out. These included the AST/ALT ratios; a value of >0.7 was considered abnormal, the APRI score was calculated via the formula [(AST/ULN)/platelet count (10^9/L)]×100; a value of >1.5 suggested liver fibrosis, and also the FIB-4 index was calculated via the formula age (yrs)×AST level/platelet count×√ALT; a value of ≤1.3 has been reported to have a 90% negative predictive value for cirrhosis. These cut-off values were based on previous studies by Siberry et al. (10), Kapogiannis et al. (11), Pokorska-Śpiewaket al. (12), Aurpibul et al. (13), Iacobellis et al. (14), and Shah et al. (15).

The relationships between abnormal AST/ALT ratios, abnormal APRI scores, and abnormal FIB-4 index values with the individual risk factors of liver dysfunction [mode of acquisition of HIV, the type of ART, duration of ART, level of immunosuppression, presence of anemia, and body mass index (BMI)] were assessed.

In cases of abnormal biomarkers of liver dysfunction, a routine ultrasound scan was performed. An ultrasound score was allotted based on the presence of 6 abnormal ultrasound variables (presence of liver enlargement, irregular liver surface, abnormal liver echotexture, blunted liver edge, the presence of splenomegaly, and dilated portal veins). Each variable was assigned a score of 1 and a total resultant score was calculated for all these patients. The presence of liver fibrosis was assessed using the allotted score.

Statistical Analysis

Data entry was performed using a Microsoft Excel sheet and analyzed statistically using SPSS software 17. Appropriate tests with a 90% confidence interval were applied. Qualitative variables were expressed as frequency and percentage. Quantitative variables were expressed as mean, median, and inter-quartile ranges. Covariates considered as potential predictors of elevated APRI were

identified using appropriate statistical tests and significance was set at a p-value of <0.05.

Results

During the study period, a total of 44 HIV-positive children were recruited into this study. Out of these 44 children, 40 (91%) had acquired HIV from their mother, 2 (5%) acquired it from a blood transfusion and 1 (2%) from an infected needle. In the one other case, the status of the parents was not known as the child was adopted by an non-governmental organizations. On clinical examination, pallor was found in 13 (30%), hepatomegaly in 12 (27%), and lymphadenopathy in 6 (14%), while the commonly seen clinical symptoms were recurrent cough (16%), recurrentdiarrhea (14%), and abdominal pain (8%). Clinically visible jaundice was found in 2 (4.5%) patients only. Out of the 44 children, 25 (56.8%) were on zidovudine, 23 (52,3%) were on efavirenz, 14 (31.8%) on abacavir, 13 (29.5%) were on nevirapine, 5 (11.4%) were on tenofovir, and 8 (18.2%) were on protease inhibitor-based regimen. Out of the 44 children, 2 (4%) were in stage IV, 6 (14%) were in stage III, 7 (16%) were in stage II, and 29 (66%) were in Stage I of the HIV illness [as per World Health Organization (WHO) classifications].

On assessing the liver function tests, the mean value of serum bilirubin was 0.55 [standard deviation (SD) 0.3], the mean serum ALT level was 31.93 (SD 21), and the mean serum AST level was 37 (SD 16). The mean value of total serum protein was 7.5 (SD 0.75) and serum albumin was 4.1 (SD 0.49). Theserum bilirubin level was elevated in only 2 patients (4.5%), serum ALT level was elevatedin 7 (16%) patients, and serum AST level was elevated in 8 patients (18%) (Table I). Out of the 7 patients who had elevated ALT, 5 patients had grade 1 hepatotoxicity and 2 patients had grade 2 hepatotoxicity, whereas all 8 patients with elevated AST had grade 1 hepatotoxicity.

Out of the 44 children, 42 (95%) had abnormal AST/ALT ratios. Out of these 42 children, 29 (69%) were males and 13 (31%) were females. There was no statistical difference between males and females in terms of abnormal ALT/AST ratios (p-value 0.57) (Table II). Fifteen out of the 16 (94%) children with anemia had an abnormal AST/ALT ratios. All 13 children who were on the nevirapine-based ART regimen had an abnormal AST/ALT ratio. The duration of ART for more than 1 year was significantly associated with abnormal AST/ALT ratios. We found that ART initiation was significantly associated with abnormal AST/ALT ratios. Seventeen out of the 18 (94.4%) children with CD4 counts of less than 500 and 25 out of the 26 (96%) children with CD4 counts of more than 500 had an abnormal AST/ALT ratio. All 9 children with BMI less than the 5th percentile had an abnormal AST/ALT ratio (Table III).

Table I. Liver function test estimates

Lab. parameters	Elevated (n) (%)	Normal (n) (%)	Mean (SD)
Serum bilirubin			
<5 years	1	3	
5-10 years	0	16	
>10 years	1	23	
Total	2 (4.5)	42 (95.5)	0.55 (0.3)
***Serum AST levels**			
<5 years	1	3	
5-10 years	4	12	
>10 years	3	21	
Total	8 (18)	36 (82)	37.52 (16)
***Serum ALT levels**			
<5 years	0	4	
5-10 years	4	12	
>10 years	3	21	
Total	7 (16)	37 (84)	31.93 (21)

*Cut-offs as per the Harriet Lane handbook 20th edition and Abnormal liver enzymes (ALT/AST) of >1.25 times the ULN (upper limit of normal).
SD: Standard deviation, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase

Out of the 44 children, 3 (7%) had abnormal APRI scores, all of these were males and 2 (5%) had a FIB-4 index of >1.3, with both of these being males. Out of the 3 children with APRI >1.5, two were more than 5 years of age. This was significant (p-value<0.001). Also, 2 out of the 3 with elevated APRI had anemia with hemoglobin less than 11 gm/dL.This was significant (p-value=0.004). Out of the 13 children on the nevirapine-based ART regimen, none had an abnormal APRI score. There were significantly more children with APRI >1.5 among those on ART for more than 1 year. Also, age at ART initiation was not significant in APRI elevation. Two out of the 18 (11%) children with CD4 counts of less than 500 and only 1 child with CD4 count of more than 500 had an abnormal APRI score. Those with CD4 counts of more than 350 cells/cumm had lower APRI scores. Low BMI (below the 5th percentile) was not significantly different in the two APRI groups (Table IV).

Only 2 (5%) children out of the 40 aged more than 5 years old had an abnormal FIB-4 index. None of the children less than 5 years had an abnormal FIB-4 index. Only 1 child out of the 16 (6.3%) children with anemia had an abnormal FIB-4 index. Out of the 13 children on the nevirapine-based ART regimen, none had an abnormal FIB-4 index. This was statistically significant. Only 1 child out of the 34 (2.9%) with an ART duration of more than 1 year had an abnormal FIB-4 index. No child with age at ART initiation of less than 5 years had an abnormal FIB-4 index. Two out of the 22 (9%) children with age at ART initiation of more than 5 years had an abnormal FIB-4 index. No child with CD4 counts of more than 350 had an abnormal FIB-4 index. Only 1 child out of the 9 (11%) children with BMI less than the 5th percentile had an abnormal FIB-4 index. This was statistically significant (Table V).

Table II. Showing abnormal biomarkers of liver dysfunction in the enrolled children (n=44)

	Males	Females	Total
AST/ALT ratios >0.7	29	13	42
APRI score >1.5	3	0	3
FIB-4 index >1.3	2	0	2

ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, APRI: AST-to-platelet ratio index, FIB-4: Fibrosis-4

Table III. Showing the relation between abnormal AST/ALT ratio (>0.7) with the risk factors of liver dysfunction in the enrolled children (n=42)

Risk factors of liver dysfunction	AST/ALT ratio >0.7 n	AST/ALT ratio <0.7 n	p-values
Age <5 yrs (n=4)	4	0	0.13
Age >5 yrs (n=40)	38	2	<0.001
Anemia Hb<11 (n=16)	15	1	0.001
Nevirapine based ART (n=13)	13	0	0.001
Duration of ART (yrs)			
<1 (n=10)	9	1	0.02
1-5 (n=18)	18	0	0.001
>5 (n=16)	15	1	0.001
Age at ART initiation (yrs)			
<5 (n=22)	21	1	0.001
>5 (n=22)	21	1	0.001
Immunosuppression			
CD4 counts <350 (n=9)	8	1	0.039
CD4 counts 350-500 (n=9)	9	0	0.004
CD4 counts >500 (n=26)	25	1	<0.001
BMI <5th percentile (n=9)	9	0	0.004

ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ART: Antiretroviral therapy, BMI: Body mass index, Hb: Hemoglobin

Table IV. Showing the relation between abnormal APRI (>1.5) with the risk factors of liver dysfunction in recruited children (n=3)

Risk factors of liver dysfunction	APRI >1.5 n	APRI <1.5 n	p-values
Age <5 yrs (n=4)	1	3	0.625
Age >5 yrs (n=40)	2	38	<0.001
Anemia Hb<11 (n=16)	2	14	0.004
Nevirapine based ART (n=13)	0	13	0.001
Duration of ART (yrs)			
<1 (n=10)	2	8	0.109
1-5 (n=18)	0	18	0.001
>5 (n=16)	1	15	0.001
Age at ART initiation (yrs)			
<5 (n=22)	1	21	0.001
>5 (n=22)	2	20	0.001
Immunosuppression			
CD4 counts <350 (n=9)	2	7	0.18
CD4 counts 350-500 (n=9)	0	9	0.004
CD4 counts >500 (n=26)	1	25	<0.001
BMI <5th percentile (n=9)	2	7	0.18

APRI: AST-to-platelet ratio index, ART: Antiretroviral therapy, BMI: Body mass index

Table V. Showing relation between abnormal FIB-4 index (>1.3) with the risk factors of liver dysfunction in the enrolled children (n=2)

Risk factors of liver dysfunction	FIB-4 index (>1.3) (%)	FIB-4 index (<1.3) (%)	p-values
Age <5 yrs (n=4)	0	4	0.125
Age >5 yrs (n=40)	2	38	<0.001
Anemia Hb<11 (n=16)	1	15	0.001
Nevirapine based ART (n=13)	0	13	0.001
Duration of ART (yrs)			
<1 (n=10)	1	9	0.021
1-5 (n=19)	0	18	0.001
>5 (n=15)	1	15	0.001
Age at ART initiation (yrs)			
<5 (n=22)	0	22	0.001
>5 (n=22)	2	20	0.001
Immunosuppression			
CD4 counts <350 (n=9)	2	7	0.18
CD4 counts 350-500 (n=9)	0	9	0.004
CD4 counts >500 (n=26)	0	26	<0.001
BMI <5th percentile (n=9)	1	8	0.039

ART: Antiretroviral therapy, FIB-4: Fibrosis-4, BMI: Body mass index, Hb: Hemoglobin

Forty-one out of the 44 (93%) children had an ultrasound score of 0-1 indicating mild or no fibrosis, which was significantly more than the 3 (7%) children with an ultrasound score of 2-3.

Co-infection with hepatitis B and/or hepatitis C-only 1 child was found to be positive for hepatitis B. No child was positive for hepatitis C.

Discussion

This cross-sectional study enrolled 44 children with HIV and evaluated them for liver dysfunction using non-invasive biomarkers of liver dysfunction and an ultrasound scoring system.

Forty out of the 44 (91%) children had perinatally acquired HIV highlighting the mother-to-child transmission of HIV. It is well known that more than 95% of pediatric HIV cases are acquired via vertical transmission. Many studies have shown the same. A study by Kapogiannis et al. (11) showed that 65% of HIV-positive children had perinatally acquired HIV infections. The study by Aurpibulet al. (13) showed 98% were perinatally infected. Studies by Siberry et al. (10) in Latin America and by Siberry et al. (16) in the United States were performed only in perinatally acquired HIV children. In two children in our study, the mother was negative and these children had acquired their infection via blood transfusion. In one case, the child was an intravenous drug user and had acquired the infection by a parenteral route.

On analyzing liver functions, we found that serum bilirubin level was elevated in only 2 (4.5%) cases, whereas ALT was elevated in 7 (16%) cases and AST was elevated in 8 (18%) cases. The levels of ALT were seen to be higher in this study compared to the study carried out on south-east Asian children (13), the possible explanation could be the variable stage of HIV and the poor nutritional status of the patients enrolled in this study. The increase in ALT levels was found to be more (32%) in South African children as 74% of those patients were in WHO stage 3 or 4 of the HIV in that study (17).

Out of the 44 children, 42 (95.5%) showed abnormal AST/ALT ratios, only 3 (7%) showed abnormal APRI scores and only 2 (4.5%) showed abnormal FIB-4 index. This was seen because most of the children had been on ART for more than 1 year at the time of recruitment. It is well known that combination ART is protective against liver enzyme elevations. This was also seen because most children were diagnosed early and started on ART early in the course of their HIV disease. The findings in our study are similar to the study by Aurpibul et al. (13) in Asian children, where after ART initiation, AST/ALT ratios >0.7 were seen in 845 out of 852 (99%) children, APRI scores >1.5 were seen in 27 out of 852 (3.2%) children, and an FIB-4 index >1.3 was seen in 6 out of 852 (0.7%) children.

We found that 15 out of the 16 (94%) children with anemia had an abnormal AST/ALT ratio, which shows the presence of anemia is related to liver dysfunction. This is because children with anemia have more advanced HIV disease, malnutrition, and concomitant infection, thus forming a vicious cycle in them and thus making them unable to compensate for the physiological stress caused by the inflammatory response to the initial treatment. This finding is similar to the study by Aurpibul et al. (13) on Asian children.

It is wellknown that liver enzyme elevations are common in HIV infections. In many HIV-positive patients with elevated liver enzymes, the elevation is not explained

by an identified underlying liver disease or toxin and thus may directly occur either due to antiretroviral drug toxicity or the HIV infection itself. Studies from developed countries have reported correlations between HIV viral load and serum aminotransferase levels in HIV-positive antiretroviral (ART)-naive patients (18). There are no similar studies from India for comparison.

However, one study conducted in Uganda found that the risk of clinically significant hepatotoxicity was low, even in HIV-positive patients on ART and among HIV/hepatitis B virus (HBV) co-infected persons. Nevertheless, there is emerging evidence that HIV infection, even in the absence of ART toxicity and other cofactors, may have a direct impact on liver fibrosis pathogenesis, and on further progression to liver disease (19,20).

Thus, it appears that children with perinatally acquired HIV develop overt liver fibrosis due to early exposure to HIV and this manifests in their adult life. During childhood, these subtle hepatic enzymes indicate an ongoing necro-inflammatory process in the liver.

In this study, we tried to explore any significant associations with elevated AST/ALT ratios. Thirty-eight children out of the 40 (95%) aged more than 5 years old had an abnormal AST/ALT ratio (p-value<0.001). Fifteen out of the 16 (94%) children with anemia had an abnormal AST/ALT ratio. All 13 children on the nevirapine-based ART regimen had an abnormal AST/ALT ratio. ART initiation was significantly associated with an abnormal AST/ALT ratio. Seventeen out of the 18 (94.4%) children with CD4 counts of less than 500 and 25 out of the 26 (96%) children with CD4 counts of more than 500 had an abnormal AST/ALT ratio. Children with better CD4 counts because of ART had significantly elevated ALT/AST ratios. All 9 children with BMI less than the 5th percentile had an abnormal AST/ALT ratio. Thus, it appears that older age, ART (especially nevirapine), and low BMI are associated with abnormal AST/ALT ratios.

Abnormal AST/ALT ratios were seen in almost all patients in this study. An AST/ALT ratio of >1 is considered significant in predicting advanced liver disease in adult patients, whereas in this study, the cut-off ratio of 0.7 was taken to be significant after being derived from similar pediatric studies. Although this test is cost-effective and easily available, it has less specificity according to various studies (21,22) carried out in the past on adult patients. However, the higher ratioused in adult studies could be considered to identify liver diseases in pediatric patients living with HIV.In a meta-analysis of 40 studies, investigators concluded that APRI scores greater than 0.7 had a sensitivity of 77% and a specificity of 72% in predicting significant hepatic fibrosis (23). The higher the value of the APRI (>1.5), the greater its positive predictive value (and its ability to rule in cirrhosis). In our study out of the 44 children, 3 had APRI of more than 1.5. Also, as thrombocytopenia is common among HIV-positive patients and platelet count is used in APRI calculation, higher APRI values in HIV-positive individuals may be due to their HIV infection rather than the underlying liver disease. Multiple factors like chronic HIV infection and thrombocytopenia contribute to negatively affect APRI scores.

There were significantly more children with APRI less than 1.5 among those who had been on ART for more than 1 year. However, age at ART initiation was not significant on APRI elevation. Those with CD4 counts of more than 350 cells/cumm had lower APRI scores. Lower BMI than the 5th percentile was not significantly different in those with elevated APRI.Thus, it appears that the initiation of ART is protective and is associated with lower APRI scores. ART was protective against liver dysfunction with studies by Kapogiannis et al. (11) in the United States, Aurpibul et al. (13) in Asia, Siberry et al. (10) in Latin America, Siberry et al. (16) in the United States, and Pokorska-Śpiewaket al. (12) in Poland showing that longer and better ART led to lower APRI scores.

Next in this study, the FIB-4 index was estimated. Only 2 children out of the 40 (5%) aged more than 5 years had an abnormal FIB-4 index. None of the children less than 5 years had an abnormal FIB-4 index. Out of the 13 children on the Nevirapine-based ART regimen, none had an abnormal FIB-4 index. This was statistically significant. No child with age at ART initiation of less than 5 years had an abnormal FIB-4 index. Two out of the 22 (9%) children with age at ART initiation of more than 5 years had an abnormal FIB-4 index. No child with CD4 counts more than 500 had an abnormal FIB-4 index showing that the immunocompetent state is associated with better control of HIV infection and thus reduces the risk of liver fibrosis. The study by Kapogiannis et al. (11) highlighted the same.

Thus, it appears that older children (more than 5 years old) with delayed ART initiation have abnormal FIB-4 index.

Hepatic echotexture was assessed in these children and a USG scoring as per Afzalet al. (24) was carried out to assess the extent of liver fibrosis. Forty-one out of the 44 (93%) children had an ultrasound score of 0-1 indicating mild to no fibrosis.

In our study, only 1 child was positive for hepatitis B and had acquired this infection through intravenous drug

use. All the biomarkers including the AST/ALT ratio, APRI, and FIB-4 were abnormal in this child. It is well known that HIV/HBV co-infection is associated with liver dysfunction. This has been highlighted in various studies by Shiferaw et al. (25) in Ethiopia, Siberry et al. (10) in Latin America, and Pokorska-Śpiewak et al. (12) in Poland.

Study Limitations

The small number of children in this study was a limiting factor.

Conclusion

It appears that chronic HIV infection is associated with hepatic dysfunction. This was reflected by abnormal AST/ALT ratios in a large number of children in this study. However, the high cut-off value for the AST/ALT ratio has to be reconsidered in children. Furthermore, elevated APRI scores and FIB-4 index in some of them signal evolving liver fibrosis. None of these were hepatitis C virus infected. Thus, HIV infection caused abnormalities in liver function through multiple pathogenetic mechanisms. A longer follow-up with a large number of children may reveal many more children with HIV-associated liver diseases.

Ethics

Ethics Committee Approval: This cross-sectional study was conducted at the ART clinic of the Department of Pediatrics at an institute located in New Delhi, India, after taking permission from the Institutional Ethics Committee (protocol no: 19/9/17, dated: 27.10.2017).

Informed Consent: Informed consent was obtainedfrom all patients included in this study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: R.K., Design: R.K., Data Collection and/or Processing: S.Y., Analysis and/or S.Y., Interpretation: S.Y., R.K., Literature Search: D.K., Writing: D.K.

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Original Article

Urine Neutrophil Gelatinase-associated Lipocalin as a Prognostic Biomarker in the First Episode of Idiopathic Nephrotic Syndrome in Children

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ABSTRACT

Aim: Idiopathic nephrotic syndrome (NS) is the most common glomerular disorder of childhood. Its prognosis is correlated with treatment responsiveness and not renal histopathology. Most of the children who suffer from NS experience multiple relapses and there is a risk of long-term drug dependence with possible side effects. Hence, there is always a need for markers to assess its long-term outcome even in steroid responders. Urine neutrophil gelatinase-associated lipocalin (uNGAL) is an early risk marker of acute kidney injury and also a marker of progression of chronic kidney disease. Our aim was to determine if urine NGAL could predict steroid responsiveness at the onset of NS, which would help in the planning and monitoring of the treatment in idiopathic NS. The aims of this study were to determine the levels of uNGAL in children who were having their first episode of NS and to study its relation with steroid resistance at 3 months.

Materials and Methods: A prospective observational study was conducted in children diagnosed with their first episode of idiopathic NS in a tertiary care teaching hospital from January, 2019 to July, 2020. Urinary NGAL measurements were conducted before starting steroids.

Results: Seventy-nine children satisfying the inclusion criteria were included in this study. Their mean age was 7.18 (±2.86) years. The male to female ratio was 1.25:1. All 63 children who had urine NGAL less than 10 ng/mL responded to the standard dose of steroids at 8 weeks and attained remission. Out of the 16 children with NGAL over 10 ng/mL, 56.3% (n=9) responded to steroids within 8 weeks (intermediate or late steroid responders) and 43.8% (n=7) were steroid resistant NS (SRNS). Urine NGAL below 10 ng/mL was associated with steroid responsiveness in the first episode of NS at 3 months ($p<0.001$).

Conclusion: Urine NGAL below 10 ng/mL is an early predictive biomarker of steroid responsiveness in the first episode of idiopathic NS.

Keywords: Neutrophil gelatinase-associated lipocalin (NGAL), idiopathic nephrotic syndrome, steroid responsiveness, biomarker, prognosis

Introduction

Nephrotic syndrome (NS) is the most common glomerular disease in children. The prevalence of NS in children is 12-16 per 100,000 individuals and the underlying cause is idiopathic in 95% of cases (1). Oral glucocorticoids form the mainstay of treatment. Invasive renal biopsy remains the standard for diagnosis of NS in adults. Unlike in adults, steroid responsiveness is a better predictor than histopathological diagnosis in the long-term prognosis of idiopathic NS in children (2). Although most children undergoing their first episode of NS respond to standard steroid therapy, many have multiple relapses, a few have drug dependence with drug side effects and 5-10% are steroid resistant. Steroid-resistant NS is associated with a 50% risk of end-stage kidney failure and poor quality of life in childhood. An early non-invasive marker to predict

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steroid responsiveness would be helpful in better planning treatment, thereby resulting in fewer side effects of steroids.

Urine neutrophil gelatinase-associated lipocalin (uNGAL) is upregulated in cases of renal injury and acts as a highly sensitive, early biomarker for acute kidney injury (3). Also, higher urine and plasma NGAL levels are associated with disease severity and progression in chronic kidney disease (CKD). Since children with steroid resistant NS (SRNS) have a greater risk for progressive CKD, the urinary NGAL levels may be higher than in those with steroid sensitive NS (SSNS) (4). Acute kidney injury induces rapid upregulation of NGAL mRNA within the thick ascending limb of Henle's loop and the collecting ducts. Following this, the accumulation of NGAL in the distal nephron causes an increase in urine NGAL levels (5). Zhang et al. (6) concluded that NGAL is a better indicator than plasma creatinine and has a satisfactory early predictive value for acute kidney injury. NGAL increases rapidly in both serum and urine after kidney tissue damage (up to 1,000-fold).

The present study was conducted to determine if there was a correlation between urine NGAL and steroid responsiveness in children who were undergoing with first episode of idiopathic NS. Being able to predict steroid unresponsiveness based on uNGAL would help to plan early alternate treatment strategies.

Materials and Methods

This study was conducted to determine the levels of urine NGAL in the first episode of idiopathic NS and its relation with steroid responsiveness. All consecutive cases of first-episode idiopathic NS in patients aged 1 to 12 years admitted between January, 2019 and July, 2020 to a tertiary teaching institution were enrolled in this study. Out of the 85 cases, 79 children satisfying the criteria of NS as per ISPN Guidelines were enrolled (7). Children with congenital NS, secondary NS, concomitant urinary tract infections, acute kidney injury, CKD, or children on nephrotoxic medications were excluded from this study. The patients were evaluated for clinical and biochemical parameters including the presence of haematuria, hypertension, the severity of edema, levels of urine NGAL, urine protein creatinine ratio, serum albumin, and cholesterol levels. Urine samples were collected from patients in the early morning before the initiation of steroid therapy.

Figure 1 shows the flow chart used in this study. Urine NGAL was measured by a commercially available ELISA kit (Elabscience®, Houston, USA) which specifically detects human urine NGAL. This ELISA kit applies to the *in vitro* quantitative determination of human NGAL concentrations in serum, plasma, and other biological fluids. The specifications of this kit include Sensitivity: 0.10 ng/mL, Detection range: 0.16-10 ng/mL, Specificity: No significant cross-reactivity or interference between human NGAL and analogues was observed, and Repeatability: Coefficient of variation is <10%. In our study, the cut-off value of uNGAL was taken as 10 ng/mL based on previous studies (8,9).

Urine samples, collected in sterile containers, were centrifuged for 20 min and the supernatant was collected into clean tubes, aliquoted, and frozen at -20 °C until the time of urine NGAL assay. Samples were thawed and mixed thoroughly just before the assay to avoid erroneous results of repeated freeze/thaw cycles.

All children were followed up to assess their outcomes at 3 months. The children were assigned into subgroups based on their initial response to steroids. Kidney Disease Improving Global Outcomes (KDIGO) guidelines were followed for definitions (10).

Initial responder; Attainment of complete remission within the initial 4 weeks of corticosteroid therapy.

Initial non-responder/steroid resistance; Failure to achieve complete remission after 8 weeks of corticosteroid therapy.

Additionally, ISKDC data suggest that an absence of response to steroid therapy at 8 weeks indicates non-response (11), but a lack of response at the completion of 6 weeks often prompts many nephrologists to pursue renal biopsy.

According to ISPN guidelines, renal biopsy was carried out on all children who failed to attain remission by 4 weeks.

We classified the children into three groups based on their steroid response as:

Early responder; If the steroid response was obtained within the first two weeks.

Intermediate responder; When the steroid response was noted between 2-4 weeks.

Late responder; When the steroid response was obtained between 4 and 8 weeks.

Steroid resistant; When there was no steroid response even after 8 weeks.

Patients were treated as per the Indian Society of Paediatric Nephrology recommendations (7,12).

Institutional ethical clearance was obtained from Human Ethics Committee of Medical College, Thiruvananthapuram (approval no: 02/38/2019/MCT, dated on 16.01.2019), and

written informed consent was obtained prior to this study. Confidentiality was ensured and maintained throughout this study.

Statistical Analysis

Statistical analyses were performed using SPSS26. All quantitative variables are expressed as mean and standard deviation and qualitative variables as proportions. Groups were compared using non-parametric Fisher's exact test and p-values <0.05 were considered significant.

Results

Seventy-nine children were included in this study. The male to female ratio was 1.25:1. The mean age was 7.18 (±2.86) years. Out of these, 30.37% (n=24) were less than 5 years old, 56.96% (n=45) were between 5 and 10 years old, and 12.65% (n=10) above 10 years of age.

Urine NGAL was measured before starting steroids. All sixty-three children out of the seventy-nine (79.7%) who had urine NGAL ≤10 ng/mL responded to standard doses of steroids by 8 weeks. This shows that 100% of children with uNGAL ≤10 ng/mL had attained remission by 8 weeks. Out of the 16 children with NGAL >10 ng/mL, 56.3% (n=9) responded to steroids and attained remission within 8 weeks [either as intermediate (n=2) or late responders (n=7)] and 43.8% (n=7) did not show any response to steroids within the 8 weeks (SRNS). Fisher's exact test was performed and this showed that there was a statistically significant relation between NGAL value ≤10 ng/mL and increased steroid response and also between values >10 mg/dL and steroid unresponsiveness with a p-value <0.001. Table I shows the comparison of SSNS & SRNS.

91.13% (n=72) patients achieved remission within 8 weeks of steroid therapy (SSNS). Among these, 82.3% (n=65) attained remission within 4 weeks. In those who attained remission, 49.4% (n=39) attained remission within 2 weeks (early responders), 32.9% (n=26) attained remission within 2 to 4 weeks (intermediate responders) and 8.9% (n=7) attained remission after 4 weeks but within 8 weeks (late responders). However, 8.9% (n=7) of the children did not attain remission within 8 weeks (SRNS). Table II shows the patient characteristics for the low NGAL and high NGAL groups.

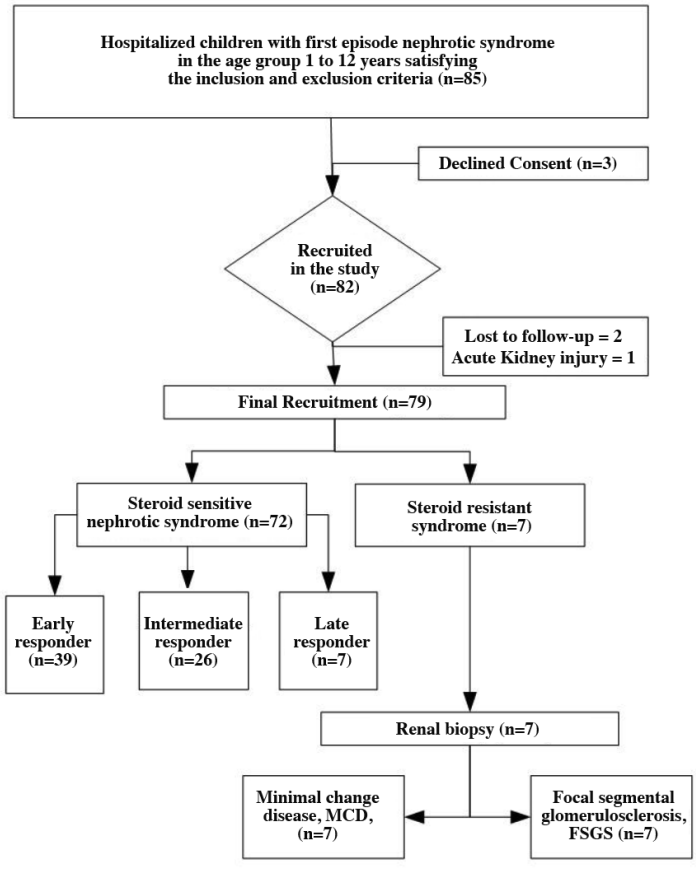

Figure 1. Flow of patients in the study

Table I. Comparison of steroid sensitive nephrotic syndrome & steroid resistant nephrotic syndrome

Parameter	SSNS (n=72) n (%)	SRNS (n=7) n (%)	p-value
Gender Male Female M:F ratio	 40 (55.6) 32 (44.4) 1.32:1	 4 (57.1) 3 (42.8) 1.33:1	 0.62
Family history (renal)	5 (6.9)	1 (14.2)	0.65
Edema Anasarca Pl effusion	 4 (5.5) 9 (12.5)	 0 1 (14.2)	 0.68 0.62
Hypertension	21 (29.1)	5 (71.4)	0.03*
Hematuria	18 (25)	5 (71.4)	0.02*
Allergy	11 (15.2)	1 (14.2)	0.71
Urine NGAL ≤10 ng/mL >10 ng/mL	 63 (87.5) 9 (12.5)	 0 7 (100)	 0.000*
Serum cholesterol <500 mg/dL ≥500 mg/dL	 60 (83.3) 12 (16.7)	 1 (14.2) 6 (85.8)	 0.008*
Serum albumin <2 g/L ≥2 g/L	 64 (88.8) 8 (11.2)	 5 (71.4) 2 (28.6)	 0.35
Biopsy MCD$^{\varsigma}$ FSGS	 --	 7 (50) 7 (50)	 --

*Statistically significant association between the study variable and SRNS
$^{\varsigma}$In 14 steroid resistant patients (Biopsy was done - 50% MCD and 50% FSGS)
NGAL: Neutrophil gelatinase-associated lipocalin, SSNS: Steroid sensitive nephrotic syndrome, SRNS: Steroid resistant nephrotic syndrome, FSGS: Focal segmental glomerulosclerosis, MCD: Minimal change disease

A renal biopsy was performed in those children (17.8%) who did not attain remission within 4 weeks as per institutional protocol. In the 14 children who underwent renal biopsy, minimal change disease was found in 50% (n=7) and focal segmental glomerulosclerosis (FSGS) was found in 50% (n=7). Of the late responders (n=7), 85.71% (n=6) were minimal change disease and 14.2% (n=1) was FSGS. Of the steroid resistant cases (n=7), 85.71% (n=6) had FSGS and 14.2% (n=1) had minimal change disease.

Discussion

NS is the most common renal disorder encountered in children and its course cannot be predicted for steroid resistance until at least after the 6th week of a steroid course. This usually paves the way for complications of steroid therapy without any clinical improvement for the patient. Renal biopsy usually arrives at a histopathological diagnosis, but it is an invasive procedure in children.

Table II. Patient characteristics between low NGAL and high NGAL groups

Parameters	NGAL≤10 ng/mL (n=63) n (%)	NGAL>10 ng/mL (n=16) n (%)	Signature
Steroid responsiveness <2 weeks 2-4 weeks 4-8 weeks >8 weeks	 39 (61.9) 24 (38.1) 0 0	 0 2 (12.5) 7 (43.8) 7 (43.8)	 <0.001*
Anasarca	4 (6.3)	0	0.39
Pleural effusion	8 (12.6)	2 (12.5)	0.67
Allergy	10 (15.8)	2 (12.5)	0.54
Hematuria	13 (20.6)	10 (62.5)	0.002*
Hypertension	13 (20.6)	13 (81.2)	<0.001*
Preterm	10 (15.8)	0	0.08
Biopsy done	0	14 (100)	<0.001*
Serum cholesterol <500 mg/dL ≥500 mg/dL	 31 (49.2) 32 (50.8)	 5 (31.2) 11 (68.8)	 0.02
Normal urea	46 (73.01)	8 (50)	0.07
Normal creatinine	34 (53.9)	9 (56.2)	0.77

*Statistically significant
NGAL: Neutrophil gelatinase-associated lipocalin

Moreover, in children, steroid responsiveness is a better prognostic marker for the disease course than renal biopsy. Therefore, if we had a non-invasive test for determining steroid responsiveness in the initial phase, we could personalize our treatment interventions in a child friendly manner. uNGAL is not only an early marker of acute kidney injury, but also a marker for progression of CKD and there is evidence in the literature about the use of this as a prognostic marker for steroid responsiveness. There are studies highlighting the role of uNGAL in SSNS, SRNS and normal children but no studies measuring uNGAL in the first episode before starting steroids. Therefore, this study was undertaken to determine whether urine NGAL could predict steroid responsiveness in the first episode of NS, which would help in the planning and monitoring of the treatment of idiopathic NS.

Among the 79 participants, 91.45% (n=72) achieved remission with steroid therapy by 8 weeks and the remaining 7 (8.9%) were steroid resistant (SRNS). In our study, 82% (n=65) of the children attained remission within the first 4 weeks of steroid therapy. This is similar to the International Study of Kidney Disease in Children (ISKDC) study and Indian study where 80% of children achieved clinical remission

within 4 weeks of corticosteroid therapy (11,12). In a study conducted by Mortazavi and Khiavi (13), 75.2% of patients responded to standard steroid therapy within 4 weeks.

In the sixteen children with an NGAL value >10 ng/mL, remission was attained in 56.3% (n=9) [intermediate (n=2) and late responders (n=7)] and 43.8% (n=7) did not attain remission. There is a significant relationship between NGAL >10 ng/mL and steroid unresponsiveness (p<0.001). In two recent studies, Bennett et al. (14,15) demonstrated the capacity of urine NGAL to predict the degree of response to steroid therapy in children with idiopathic NS, allowing health care professionals to discriminate between steroid-sensitive and steroid resistant children. There was a significant positive relationship between increasing uNGAL levels and the severity of disease, as measured by eGFR (14). In a study by Bennett et al. (15), urine NGAL is markedly increased in those patients with SRNS versus SSNS patients (in relapse or in remission of proteinuria), and versus healthy controls (p<0.001) and uNGAL also showed a high discriminatory power (AUC 0.91, p<0.0001) between SRNS and SSNS patients. In a study by Nickavar et al. (5), in 52 children with idiopathic NS (n=27 were steroid resistant; and n=25 were steroid responsive) aged from 1 to 16 years, urine NGAL was significantly higher in the steroid resistant patients in comparison to the steroid sensitive patients and they considered uNGAL to be a marker of steroid resistance in children with idiopathic NS.

Additionally, several previous studies have demonstrated that urine NGAL concentrations are not affected by age or gender in the paediatric population, lending support to the conclusion that the elevated urine NGAL levels seen in the SRNS children were not influenced by these factors (16-18).

In a study by Cangemi et al. (8), the calculated limits of blank (LOB) and detection (LOD) values were 0.5 ng/mL and 0.95 ng/mL, respectively. The distribution of uNGAL values approximated a log-normal distribution (median 5.2 ng/mL, interquartile range 2.5-12.8 ng/mL) (17). Another study by Bennett et al. (9) revealed a median of 6.6 ng/mL with IQR 2.8 to 17 ng/mL (8).

Nishida et al. (19) measured serum and urinary NGAL levels in children with renal diseases such as renal dysfunction (estimated glomerular filtration rate <90 mL/min 1.73 m^2), proliferative glomerulonephritis, steroid-resistant NS, steroid-sensitive NS, and tubular dysfunction.

They found that both serum and urinary NGAL levels showed significant inverse correlations with an estimated glomerular filtration rate in the analysis with all subjects, and also in the analysis with the renal dysfunction group. Additionally, in those patients with tubular dysfunction, the increase of the urinary NGAL level was remarkable compared with the other disease groups (19). This is in line with our hypothesis as SRNS is associated with a greater risk of progression and increased tubular damage, resulting in the excretion of low molecular weight proteins such as NGAL in urine. The elevated levels of urinary NGAL represent a "real-time" indicator of active inflammation and tubular injury with ongoing proteinuria (4,20,21).

Mishra et al. (22) showed that uNGAL had significant positive correlations with the duration of illness (r=0.342, p=0.006), the urine protein creatinine ratio (r=0.594, p<0.001), and a negative correlation with serum albumin (r=0.470, p<0.001) and their conclusion was that the uNGAL/creatinine level correlated with the activity of the disease and it can distinguish not only SRNS from SSNS, but also FSGS and minimal change disease histopathological sub-types of SRNS in children. In our study, all children had cholesterol values above 200 mg/dL. Among the 18 children who had serum cholesterol ≥500 mg/dL, 31.2% (n=6) did not achieve remission, whereas 68.8% (n=11) attained remission. There was a statistically significant association between very high serum cholesterol ≥500 mg/dL and steroid unresponsiveness (p=0.008). This is similar to the observations made by Krishanamurthy et al. (23) that serum cholesterol in SRNS cases shows statistically significant elevation compared to other types.

In the study group, haematuria was present in 27% (microscopic) and 73% of the children did not have haematuria. This is similar to a study carried out by the international kidney disease foundation which showed that microscopic haematuria can present in 20% of cases, while macroscopic haematuria is rare in idiopathic NS (11). There is a statistically significant relation between haematuria and steroid unresponsiveness with a p-value of 0.002. A similar observation was made in a study conducted by Mortazavi and Khiavi (13) where patients with SRNS had a higher frequency of haematuria (p=0.001) and higher mean age (p=0.017) compared with the SSNS group.

Among our study group, 33% of the children were found to have hypertension at admission, whereas 67% of children had normal blood pressure. In a study conducted by Tapia and Bashir (24), moderate arterial hypertension was present in 25% of cases. In our study, of the 26 children who had hypertension, 21/72 (29%) were in the steroid sensitive group and 5/7 (71%) were in the steroid resistant group. A statistically significant association between the presence of

hypertension and steroid unresponsiveness was observed with a p-value of 0.03. A similar study conducted by Manasa et al. (25) also showed a significant association between hypertension and steroid unresponsiveness with a p-value of 0.0001.

Study Limitations

Our study was from a single centre, with a short-term follow-up of 3 months. Additionally, we did not follow-up these children to find out whether those with initial steroid response developed steroid resistance in subsequent relapses. This study was performed with an ELISA kit with a detection range of 0.1 to 10 ng/mL only. Values above 10 ng/mL could not be measured as a quantitative figure in our study, which was a major limitation.

Conclusion

A single measurement of urine NGAL in the first episode of NS before starting treatment helps to predict steroid responsiveness. Higher urine NGAL levels (>10 ng/mL) are seen in late steroid responders and steroid-resistant NS. Larger cohort studies with a longer duration of follow-up are required to objectively assess the role of urinary NGAL levels in the first episode in predicting the course of idiopathic NS.

We could use this study model to determine whether higher NGAL levels predict steroid responsiveness in the initial phase but could not utilize it as a marker for difficult NS including frequently relapsing NS or steroid dependent NS, since we only followed up the children for 3 months. The quantitative estimation, though costlier, may provide better understanding. But, screening by a semi-quantitative (cut-off value of uNGAL being 10 ng/mL) test as used in this study may be useful in resource-limited centres. Although the gold standard is renal biopsy, it is an invasive procedure. Therefore, the utility of markers such as urine NGAL coupled with predictive clinical variables such as hypertension and haematuria may provide the physician with valuable information. A more child-friendly personalized evaluation and subsequent treatment strategies can be planned based on screening tools such as urinary uNGAL.

Ethics

Ethics Committee Approval: Institutional ethical clearance was obtained from Human Ethics Committee of Medical College, Thiruvananthapuram (approval no: 02/38/2019/MCT, dated on 16.01.2019).

Informed Consent: Written informed consent was obtained prior to this study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: S.J., S.U., G.S., Concept: G.P., S.J., Design: G.P., S.U., G.S., Data Collection or Processing: G.P., S.U., Analysis or Interpretation: G.P., S.J., S.U., G.S., Writing: G.P., S.J., G.S.

Conflict of Interest: No conflict of interest is declared by the authors.

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Original Article

Resveratrol Supplementation Attenuates Excessive Inflammation and Helps Restore Impaired Restitution in an Intestinal Epithelial Cell Culture Model

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ABSTRACT

Aim: Sustained release of inflammatory mediators, excessive inflammatory response and impaired intestinal epithelial restitution are well-known mechanisms in the pathogenesis of necrotizing enterocolitis. This study focused on the effect of resveratrol on these pathways.

Materials and Methods: In this study, the rat crypt intestinal cell line (IEC-6) culture, an application of lipopolysaccharide or a cytokine mixture and a scratch migration assay model were used. Nitric oxide synthase-2 (iNOS) and cyclooxygenase-2 (COX-2), focal adhesion kinase (FAK) and its phosphorylated form (pFAK) levels were assessed.

Results: IEC-6 cells covered 88% of the denuded area in the control, 54% in LPS, and 35% in cytomix groups at the 24th hour. The treatment with resveratrol at doses of 0.5, 1 and 5 µM/L before LPS resulted in the repair of 84%, 87% and 76% of the denuded areas, respectively. Likewise, with cytomix, it was 86%, 82%, and 78%. Resveratrol at a dose of 5 µM/L prevented an increase in iNOS levels. All three doses of resveratrol were effective in preventing increases in COX levels. FAK or pFAK expressions remained unchanged in all groups.

Conclusion: Resveratrol, being known for its antioxidant features, suppresses excessive inflammatory response and helps preservation of mucosal integrity by conservation of epithelial restitution.

Keywords: Necrotizing enterocolitis, resveratrol, restitution, inflammation, nitric oxide synthase-2

Introduction

Necrotizing enterocolitis (NEC) is a life-threatening condition mainly effecting premature infants in neonatal intensive care units. An inflammatory response within the bowel wall associated with a sustained release of inflammatory mediators results in impairment of microcirculation leading to a spectrum of ischemic changes in the bowel, ranging from focal mucosal injury to total ischemia of the whole bowel. Despite an overwhelming number of experimental and clinical studies in the literature, its etiopathogenesis still remains elusive (1).

NEC is a disease characterized by a systemic inflammatory response initiated by the intestinal mucosal immune system and a resultant disruption in the integrity of gut mucosal barrier (1). The exact mechanisms triggering this cascade are still unknown despite numerous studies focusing on this

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excessive inflammation and impairment in mucosal healing (2,3). For a long time, encouraging breastmilk feeding was the only prevention strategy with proven effectiveness (1,4,5). Recently, probiotics have been shown to be effective by various studies including several meta-analyses. However, there is still not enough satisfactory data to recommend the use of any specific probiotic strain (6).

Resveratrol is a well-known phytoalexin, mostly cited due to its anti-inflammatory and vasorelaxant features. It has been widely investigated in a wide spectrum of diseases including various cancers, cardiovascular diseases, Alzheimer's disease, and different cascades of inflammation (7). Therefore, we hypothesized that resveratrol may also be beneficial in preventing NEC regarding its effects on various inflammatory pathways. In a previous study by our group, the protective effect of resveratrol was remarkable in an animal model (8). Its dietetic supplementation to newborn rats prevented 'nitric oxide synthase-2 (iNOS)' expression and morphologic changes in an experimental NEC model. In this current study, we aimed to further dissect the mechanisms with which resveratrol prevents excessive inflammation and impaired restitution in an IEC-6 cell culture model.

Materials and Methods

Cell Culture and Treatments

The rat crypt intestinal cell line, IEC-6 cells, were maintained as per recommendations of the manufacturer at 37 °C and 5% CO_2. The tissue culture medium consisted of a combination of Roswell Park Memorial Institute medium (45%), Dulbecco's Modified Eagle's Medium (4.5 g/L glucose: 45%), and heat inactivated fetal bovine serum (10%). The medium also contained 100 µg/mL streptomycin, 100 U/mL penicillin, 4 mM L-glutamine, and 0.1 U/mL insulin. Cells at the passages 15-20 were used for these experiments. Ethanol was used as the vehicle for resveratrol.

All experiments were held in two groups to reproduce the inflammatory environment. Lipopolysaccharide (LPS) at a dose of 50 µg/ml was applied in the first, and a mixture of cytokines consisting of TNF a (10 ng/mL), IFN γ (100 ng/mL) and IL-1β (1 ng/mL) was applied in the second sets of experiments (9-11).

Wound Healing Assay

Two perpendicular lines with an intersection at the center of the well were drawn at the outside bottom of the six-well plates before the passaging of the cells. Experimental wounds were made with yellow-tip pipette parallel to the vertical line to ensure the same area was photographed each time (11).

Each six-well plate was configured as follows; one well as the "control", one well for "treated only with ethanol", one for either "LPS" or "cytomix", and three wells with different doses of "resveratrol" followed by LPS or cytomix application. At least three sets of experiments were performed for each group. The cells in the control group were treated with serum-free medium alone.

The experiments started with 12 hours of serum starvation. Linear wounds were created with yellow-tip pipettes. Three wells were treated with three different doses of resveratrol for one hour. Dose response studies with resveratrol at dosages of 0.1, 0.5, 1, 5 and 10 µM/L were performed. Following this, LPS or cytomix (9-11) were applied to these three wells and one non-treated well for six hours. The medium for cells was used as the vehicle both for LPS and cytomix.

Assessment of migration started with the application of LPS or cytomix. It was monitored with serial photographs of the denuded area taken at 0, 2, 4, 6, 8, 10, 12 and 24 hours under an inverted microscope (Olympus Optical, Tokyo, Japan).

Western-Blot Analysis

The multiple scrape model to reproduce the conditions of the migration assay was performed (12). For this, cells were grown in 75 cm^2 flasks to reach confluence. The same experimental groups as in the migration model were constituted. At least three sets of experiments for each group were performed. Wound healing assay revealed a statistically significant difference between groups starting at the 12^{th} hour. Therefore, cells were exposed to various treatments for 12 hours before sample collection.

iNOS and COX-2 (cyclooxygenase-2) expressions were calculated to assess the inflammatory response. The primary antibodies were rabbit polyclonal iNOS at 1:2,000 dilution, rabbit polyclonal COX-2 at 1:1,000 dilution, and mouse monoclonal ß-actin at 1:20,000 dilution. Focal adhesion kinase (FAK) and its phosphorylated form (pFAK) were assessed in order to reveal a possible pathway for their effect on migration. FAK phosphorylated from the tyrosine residue 397 ($pFAK^{397}$) was used. Both FAK and $pFAK^{397}$ were rabbit polyclonal and at 1:1,000 dilution. A horseradish peroxidase conjugated mouse/rabbit (according to the primary) antibody was used as the secondary antibody at a dilution of 1:5,000 for iNOS and COX-2, 1:3,000 for FAK and pFAK, and 1:10,000 for β-actin. The medium was

removed after incubation, and SDS-PAGE was performed as previously described (11). Protein concentrations were measured via the Lowry method (13). Bands representing the proteins were visualized using a commercially available chemiluminescence detection kit (ECL Plus; Amersham, GE Healthcare) and images were obtained using a Fusion Solo S imaging system (Vilber, France).

Materials

IEC-6 cells were obtained from DSMZ®, ACC 111 (Leibniz Institute, Braunschweig, Germany). Rabbit polyclonal iNOS antibody, monoclonal anti-β-actin, and secondary antibodies (antimouse IgG for β-actin and antirabbit IgG in goat for the others) were from SIGMA (St. Louis, Missouri, USA). Rabbit polyclonal cyclooxygenase-2 (COX-2) was from ABCAM (Biotech Lifesciences, Cambridge UK), FAK and phosphorylated FAK (pFAK) were from Invitrogen (Waltham, Ma, USA). The nitrocellulose membrane used was Hybond-ECL (Amersham, GE Healthcare, Piscataway, NJ, USA). All ingredients of the culture medium were from SIGMA.

Data Analysis

The closure of the wound, seen in repeated photographs, was measured using 'ImageJ' software (14). The photographs were transferred to ImageJ, a fixed rectangle (with the same width and length) was drawn with one edge on the horizontal marking. The denuded area in this constant rectangle was calculated using the freehand tool in each image (11).

The quantitative analysis of the Western-blot bands was performed using the Fusion Solo S software (Vilber, France). Data are given as the ratio of each protein versus β-actin band density.

The variables were investigated using histograms and Kolmogorov-Smirnov test in order to determine whether they were normally distributed. Repeated measurements ANOVA was used for statistical analysis and $p<0.05$ was accepted as significant. Variances were accepted homogeneous and a pairwise post-hoc test (LSD) was used when an overall difference was observed.

Results

Dose Titration Studies

Repeated cell subcultures and cell counting with trypan blue showed that the doubling-time for IEC-6 cells at 15-20 passages was around 50 hours. Resveratrol or its solvent ethanol alone had no effect on cell migration or on the expression of the proteins (data not shown). Dose titration studies with resveratrol at dosages of 0.1, 0.5, 1, 5, and 10 μM/L were first performed and the effective doses needed in order to prevent the effects of LPS or cytomix on migration were found to be between 0.5-5 μM/L and the remaining experiments were performed with doses of 0.5, 1, and 5 μM/L (data not shown).

Wound Healing Assay

"Intestinal restitution is impaired, and resveratrol prevented this impairment"

Representative photographs of the wounds documenting the difference in migration capacity between the groups are shown in Figure 1.

Wound closure was slower from the start in the LPS group compared to all other groups. A statistically significant difference started at the 6th hour for the dose of 0.5 μM/L and at the 12th hour for the remaining groups. Finally, IEC-6 cells covered 88% of the denuded area in the control group, 54% in the LPS group, and 84%, 87%, 76% for the resveratrol+LPS groups at doses of 0.5, 1, and 5 μM/L, respectively (Figure 2a).

Similarly, wound closure was slower with the cytomix group. The difference between the cytomix and all other groups was statistically significant starting from the 6th hour. Eventually, 35% of the denuded area was repaired in the cytomix groups and 86%, 82%, and 78% for the resveratrol + cytomix groups at doses of 0.5, 1, and 5 μM/L, respectively (Figure 2b).

Both the LPS and cytokine mixture were found to significantly impair migration and resveratrol was found to prevent this impairment.

Western Blot Analysis

"iNOS and COX-2 are elevated, and resveratrol prevented this alteration"

Both LPS and cytomix were found to increase iNOS and COX-2 expression. Resveratrol at doses of 0.5 and 1 μM/L did not prevent the increase in iNOS levels after LPS or cytomix administration but it was found to be effective at a dose of 5 μM/L (Figure 3). On the other hand, all three doses of resveratrol were found to be effective in preventing the increase in COX levels after LPS or cytomix (Figure 4).

"Total FAK expression or FAK phosphorylation (pFAK) remained unchanged in our experimental model"

Neither LPS nor cytomix were found to alter total FAK or pFAK levels, and there was no significant change with resveratrol (Figures 5 and 6). The pFAK/FAK ratio also did not change (data not shown).

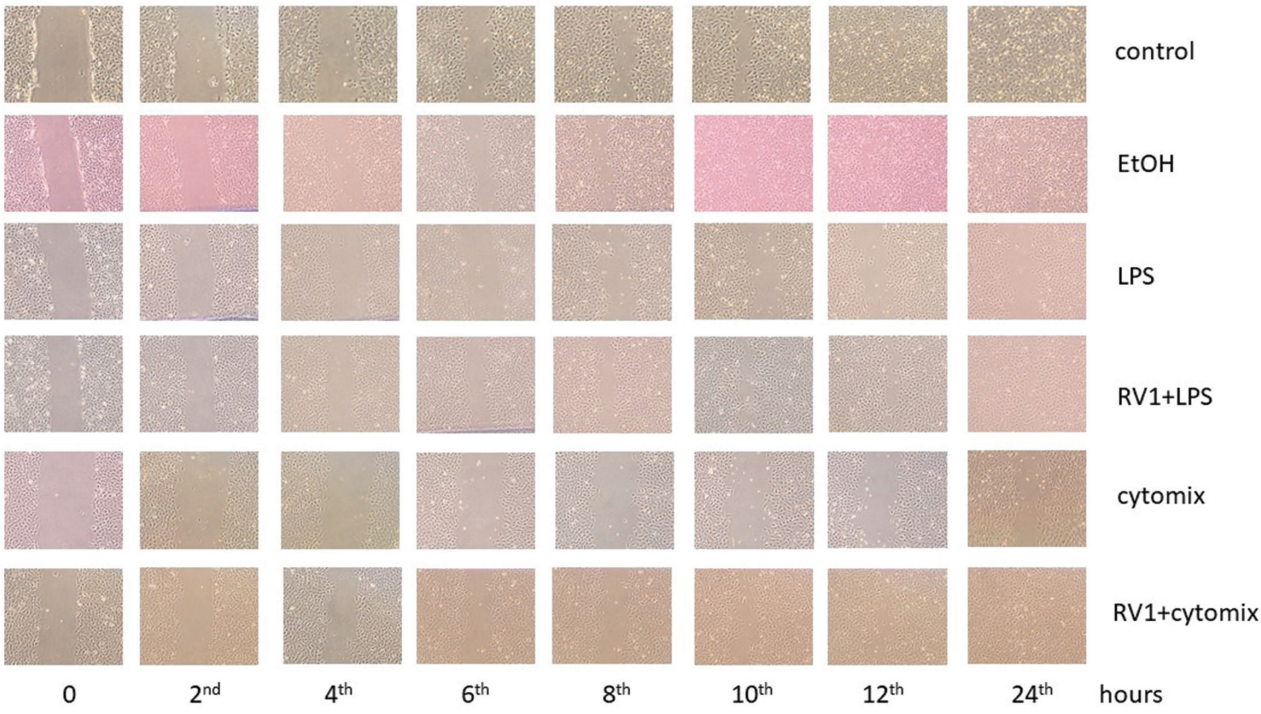

Figure 1. Representative images of the denuded area photographed for 24 hours. (EtOH: ethanol, LPS: lipopolysaccharide, RV1+LPS: treated with 1 µM/L resveratrol followed by LPS, RV1+cytomix: treated with 1 µM/L resveratrol followed by cytomix)

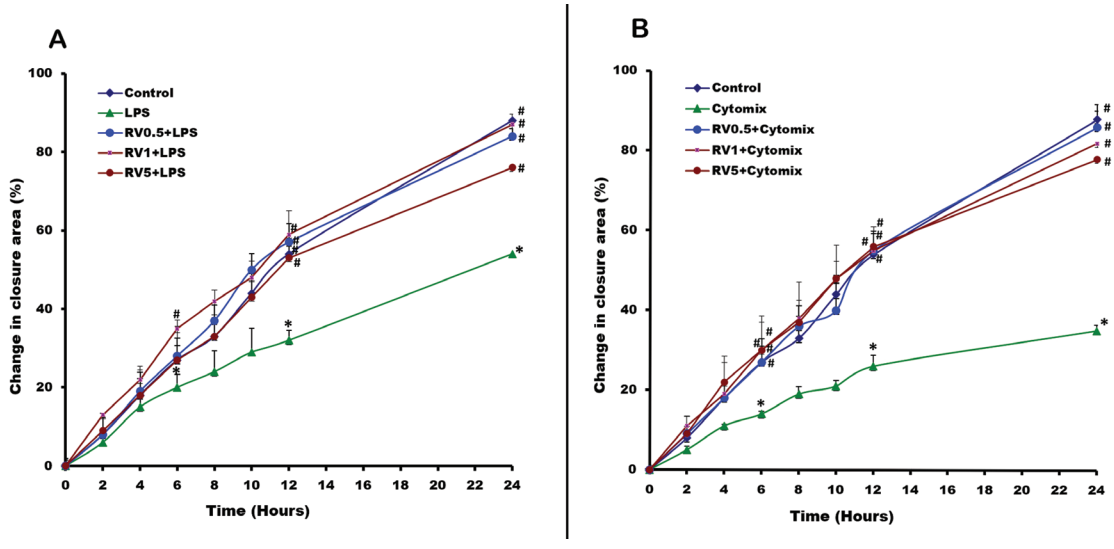

Figure 2. Time-response curves of wound closure (%) # represents statistically significant difference from the control and * from the lipopolysaccharide (LPS) or cytomix groups. A= Groups treated with LPS; B= Groups treated with cytomix

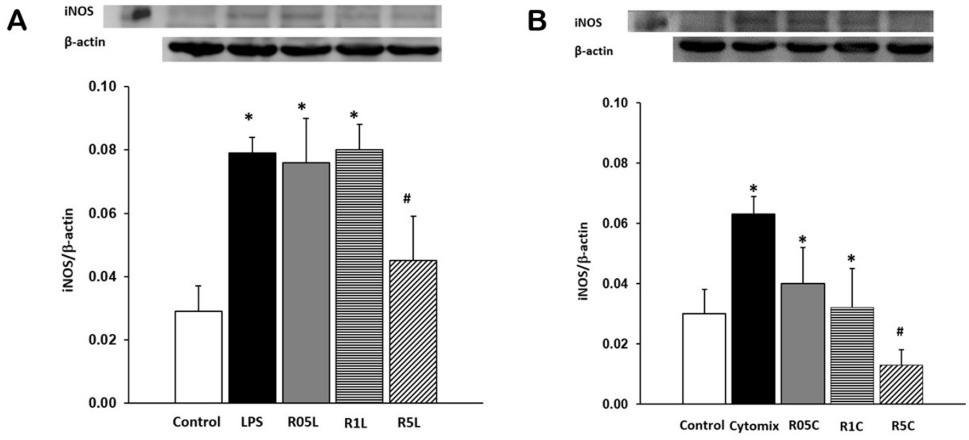

Figure 3. Western blot analysis of iNOS expression in: A= Groups treated with LPS; B= Groups treated with cytomix (LPS= lipopolysaccharide; RO5L= treated with 0.5 μM/L resveratrol followed by LPS; R1L= treated with 1 μM/L resveratrol followed by LPS; R5L= treated with 5 μM/L resveratrol followed by LPS; RO5C= treated with 0.5 μM/L resveratrol followed by cytomix; R1C= treated with 1 μM/L resveratrol followed by cytomix; R5C= treated with 5 μM/L resveratrol followed by cytomix)

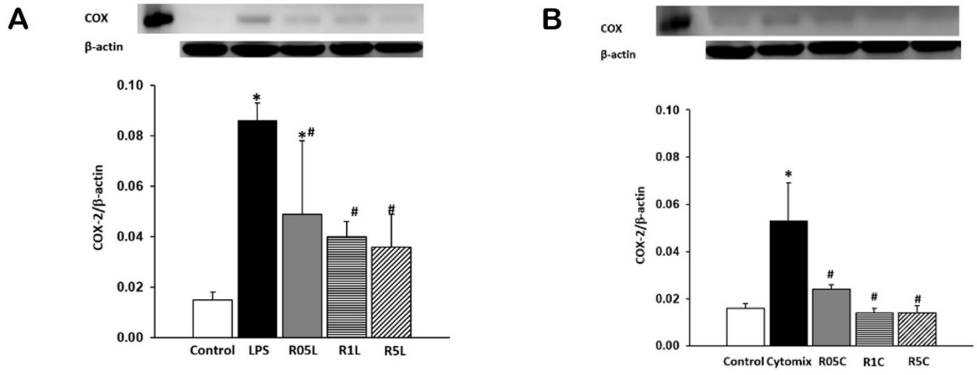

Figure 4. Western blot analysis of COX expression in: A= Groups treated with LPS; B= Groups treated with cytomix (LPS= lipopolysaccharide; RO5L= treated with 0.5 μM/L resveratrol followed by LPS; R1L= treated with 1 μM/L resveratrol followed by LPS; R5L= treated with 5 μM/L resveratrol followed by LPS; RO5C= treated with 0.5 μM/L resveratrol followed by cytomix; R1C= treated with 1 μM/L resveratrol followed by cytomix; R5C= treated with 5 μM/L resveratrol followed by cytomix)

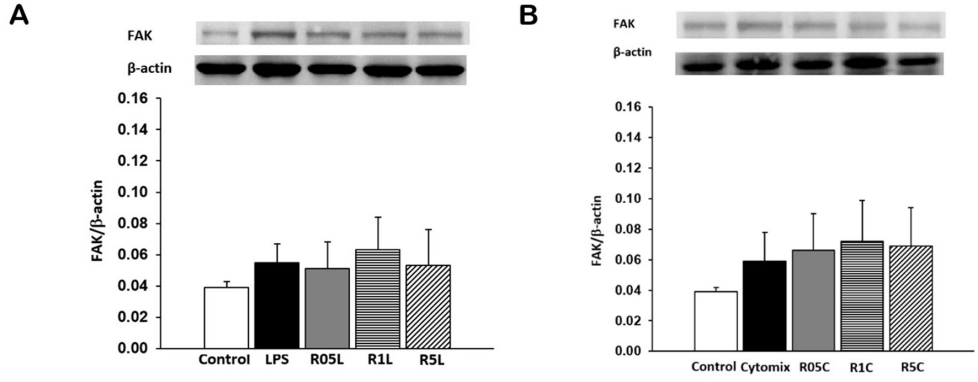

Figure 5. Western blot analysis of FAK expression in: A= Groups treated with LPS; B= Groups treated with cytomix (LPS= lipopolysaccharide; RO5L= treated with 0.5 μM/L resveratrol followed by LPS; R1L= treated with 1 μM/L resveratrol followed by LPS; R5L= treated with 5 μM/L resveratrol followed by LPS; RO5C= treated with 0.5 μM/L resveratrol followed by cytomix; R1C= treated with 1 μM/L resveratrol followed by cytomix; R5C= treated with 5 μM/L resveratrol followed by cytomix)

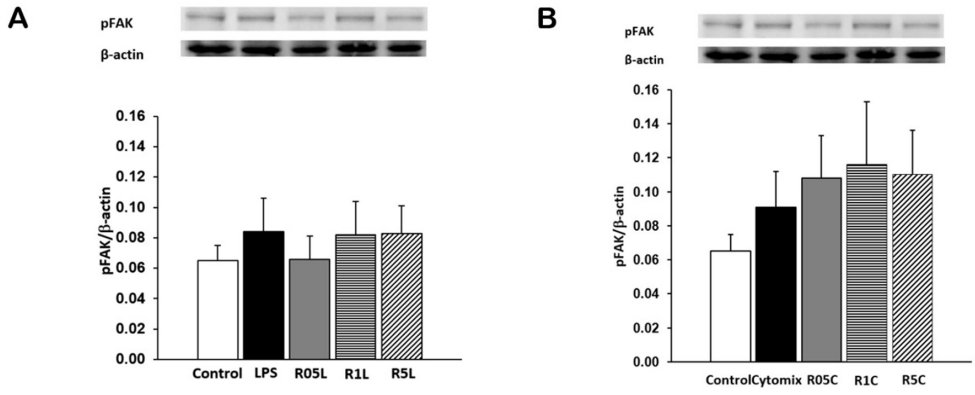

Figure 6. Western blot analysis of pFAK expression in: A= Groups treated with LPS; B= Groups treated with cytomix (LPS= lipopolysaccharide; RO5L= treated with 0.5 μM/L resveratrol followed by LPS; R1L= treated with 1 μM/L resveratrol followed by LPS; R5L= treated with 5 μM/L resveratrol followed by LPS; RO5C= treated with 0.5 μM/L resveratrol followed by cytomix; R1C= treated with 1 μM/L resveratrol followed by cytomix; R5C= treated with 5 μM/L resveratrol followed by cytomix)

Discussion

NEC is a disease characterized by a systemic inflammatory response triggered by the intestinal mucosal immune system activated by microbial antigens and enteral feeding (15). After stimulation of the mucosal immune system, an increase in the production of pro-inflammatory cytokines and some inflammatory enzymes such as COX-2 and iNOS synthase occurs. LPS and this excessive inflammation induce intestinal epithelial cell injury and enterocyte apoptosis, resulting in defects in gut mucosal integrity, followed by bacterial translocation and sepsis (1,15).

Healing of the mucosal injury starts with the migration of enterocytes, which is called restitution. In NEC, LPS and inflammatory cytokines are also shown to impair restitution and therefore the healing of mucosal defects as well as the initial injury to the immature intestine (3). Therefore, suppression of inflammatory cytokines and the preservation of the ability of enterocyte migration has a critical role in the prevention and treatment of this highly fatal disease.

Phytoalexins are antimicrobial substances produced *de novo* by plants. Some of them also have antioxidant features. Resveratrol (3,4',5 trihydoxystilbene) is a phytoalexin produced by some spermatophytes in response to injury (7). Resveratrol is a free-radical scavenger and a modulator for some fundamental enzymes in the cell cycle. Many studies have revealed its antioxidant, anti-inflammatory, anti-mutagenic, vasorelaxant, anti-aggregant and hepato-protective features (7). The previous animal model study carried out in our department revealed a remarkable protective role of resveratrol against NEC. With this current study, we aimed to investigate the possible pathways which may explain the mechanism of action and provide insights for future studies.

Previous studies have shown that sustained overexpression of intestinal iNOS plays a critical role in the pathogenesis of NEC by inducing enterocyte apoptosis with resultant intestinal barrier failure (1,15). Pro-inflammatory cytokines such as COX-2 are also known to be over-expressed in the bowel with NEC (1). We therefore hypothesized that resveratrol may prevent the exaggerated inflammatory state by suppressing the overexpression of iNOS (16) and cytokines such as COX-2 (17) due to its antioxidant and anti-inflammatory features. We used COX-2 in our experiments as it is one of the most widely investigated cytokines in NEC pathogenesis (1) and also the inhibitory effect of resveratrol on COX-2 has been shown (7,17).

Our study provided evidence that resveratrol is effective in suppressing excessive iNOS and COX-2 production caused by both LPS and/or inflammatory cytokines. Although a variety of pathways are most likely active in NEC pathogenesis, resveratrol was shown to be effective in regulating anti-inflammatory cascades.

Our second hypothesis was that resveratrol could also improve mucosal healing by restoring the migration capability of enterocytes. The second part of our study therefore focused on restitution. Intestinal epithelial cells have an impressive capacity to repair mucosal defects. This relies on the migration, proliferation, and differentiation capability of intestinal crypt cells. Before the much slower proliferation and differentiation phases, viable cells bordering the damaged area migrate to cover any defects.

This process is called restitution and it is known to be accomplished within 15 to 60 minutes (18). Migration and therefore restitution have been shown to be impaired in NEC in both ***in vivo*** and ***in vitro*** experimental studies (15).

In our study, we initially performed a time-course analysis in order to determine and confirm the doubling time of IEC-6 cells, and this doubling time was found to be around 50 hours at 15-20 passages, which was consistent with the manufacturers statement. Therefore, closure of the defects was a result of cell migration (restitution) rather than the doubling of the IEC-6 cells.

Having seen its effects on epithelial restitution, we then investigated a possible pathway which could alter this directional motility. FAK is an important regulatory protein which can modify the migration process, leading either to the formation or turnover of focal contacts. FAK protein levels or their regulation by phosphorylation (pFAK) have been asserted to be associated with the modulation of intestinal epithelial restitution (12,19). Cetin et al. (19) have shown the inhibition of intestinal restitution by endotoxin through increased focal adhesions and the increase in pFAK expression in enterocytes caused by nitric oxide with a consequent increase in the formation of focal adhesions in an experimental NEC model (20). We therefore investigated any relationships between resveratrol and total FAK expression or its phosphorylation. The tyrosine residue 397 ($pFAK^{397}$) is known to be the major site of auto-phosphorylation and has been linked with some important pathways including migration (21). However, we were unable to show any alteration in FAK or pFAK levels in our experimental cell culture model. The former study by Cetin et al. (20) was an animal model. One plausible explanation could be the association of other ***in vivo*** factors which might be responsible for FAK induction which were not represented in our cell culture model.

There is a wide range for the doses of resveratrol in the literature, and treatment with lower doses are mostly attributed to exhibit its anti-inflammatory and antioxidant effects (22); thus, dose titration studies were performed using 0.1, 0.5, 1, 5, and 10 µM/L of resveratrol. The doses 0.1 and 10 µM/L were then discarded due to poor response. Another interesting finding of our study were the better responses in migration with lower doses, and the better responses in iNOS reduction with the higher doses we used. The reduction in COX-2 was similar across all three doses. These discordant results support the presence of other possible ***in vivo*** pathways involved. Both our previous ***in vivo*** data (8) and our current ***in vitro*** data favors the possible protective role of resveratrol in NEC.

Study Limitations

This study was performed in order to evaluate the possible protective effects of resveratrol on NEC. ***In vitro*** studies cannot represent the actual disease process as there are several other pathways involved. Also, our findings do not claim any causative effect. Further studies looking into other possible pathways, studies involving chemical inhibitors or genetic modifications to solidify any causative effect, and clinical studies to support these results are required.

Conclusion

Resveratrol, being known for its antioxidant and vasorelaxant features, was found to regulate the overwhelming inflammatory response and help maintain mucosal integrity by the conservation of epithelial restitution. We found an association with iNOS and COX, but none with FAK or its phosphorylation. Other pathways should also be explored in order to fully explain its possible protective role, which was demonstrated in our previous NEC animal model.

Ethics

Ethics Committee Approval: Ethics committee approval is not required as it is a cell culture study.

Informed Consent: Informed consent is not required as it is a cell culture study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: S.T., A.E., M.O.E., Design: S.T., A.E., M.O.E., Data Collection or Processing: S.T., A.E., Analysis and Interpretation: S.T., A.E., M.O.E., Literature Search: S.T., Writing: S.T.

Conflict of Interest: The authors declared that there were no conflicts of interest.

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Original Article

Impact of the COVID-19 Pandemic on Inherited Metabolic Diseases: Evaluation of Enzyme Replacement Treatment Adherence with Telemedicine

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ABSTRACT

Aim: During the coronavirus disease-2019 (COVID-19) pandemic, visiting the hospital and getting regular infusions can be difficult for patients with chronic illnesses. Telemedicine may offer a good option for the management of chronic diseases such as lysosomal storage diseases (LSD).

Materials and Methods: LSD patients at the Unit of Metabolic Diseases of Ege University were contacted by phone between April, 2020 and March, 2021 during the COVID-19 pandemic. Telemedicine appointments were performed at intervals every month or three months, depending on the patients' compliance with their treatment.

Results: Ninety-two LSD patients [Mucopolysaccharidosis (MPS) I, MPS II, MPS IVA, MPS VI, MPS VII, Gaucher, Fabry, and Pompe] were included in this study. The total skipped treatment rate within one year was 17.1%. Most of the months of interruption were consonant with the time of social isolation. The treatment interruption in patients under 18 years was lower than in patients over 18 years. A positive correlation was detected between the age of patients and the interruption of treatment.

Conclusion: The curfew periods might be one of the causes of missed treatment sessions. Telemedicine is a good method to improve the continuity of treatment. This study showed that the number of interrupted enzyme replacement treatments could be decreased via ongoing telemedicine appointments.

Keywords: Inherited metabolic diseases, Gaucher, Fabry, COVID-19, enzyme replacement treatment, telemedicine

Introduction

Coronavirus disease-2019 (COVID-19), caused by the severe acute respiratory syndrome-coronavirus-2 virus, was declared a global epidemic by the World Health Organization in March, 2021. The high morbidity and mortality rates of the COVID-19 infection are known (1). The Turkish Government implemented intermittent lockdowns to limit the spread of COVID-19 starting in March, 2020 (2). During the COVID-19 pandemic, going to the hospital can be difficult for patients with chronic illnesses (3). To minimize disruption in health services, in some countries, a telemedicine application was started for chronic disease patients who could not come to health institutions due to the pandemic (4).

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Lysosomal storage diseases (LSD) have multi-organ involvement due to the accumulation of toxic substances in the lysosome. This condition can produce morbidity and mortality. Intravenous enzyme replacement therapy (ERT) is given to prevent the accumulation in some types of LSD. It should be applied regularly to get the maximum benefit from the treatment (5).

During the COVID-19 pandemic, many patients did not visit the hospital for fear of infection (3,6,7). Therefore, compliance in getting regular ERT infusions may be insufficient (8). Increasing the patient's motivation for compliance with the treatment by communicating via the telemedicine method may be a good option for the treatment management of these patients (9,10). In this study, we aimed to evaluate the compliance of LSD patients to treatment using the telemedicine method.

Materials and Methods

LSD patients who were followed up in the Ege University Faculty of Medicine, Department of Pediatric Metabolism and Nutrition, received ERT. Adherence to ERT was evaluated via telemedicine interviews between April, 2020 and March, 2021. The telemedicine appointments were performed by means of telephone calls to the patients. Telemedicine was performed at certain intervals according to the rate of treatment adherence. We performed a telemedicine appointment every month if the patient did not follow their treatment schedule properly, or when the physician deemed necessary. In those patients who were receiving regular ERT, telemedicine was performed at three-month intervals. Patients were advised not to interrupt their treatment during every telemedicine interview.

During telemedicine appointments, patients were questioned as to whether they had interrupted their treatment. Patients who did not comply with the therapy schedule were advised to follow the treatment program regularly. No other recommendations were made to the patients regarding their medical conditions.

All procedures followed were to the ethical standards of the Local Ethics Committee of Ege University (21-11.1T/43), and the Helsinki Declaration (2013). All patients or their parents included in this study gave informed consent to take part in this study.

The interruption of treatment percentages was calculated according to the number of infusions. The mean, standard deviation, median, minimum, maximum, frequency, and ratio values were calculated with the percentages of missing treatment and these were used in the descriptive statistics. The minimum percentage (0%) was defined as patients who had missed all their infusions. Patients who had completed all their treatments were defined as maximum percentage (100%).

Statistical Analysis

The distribution of variables was evaluated with the Kolmogorov-Smirnov test. The Mann-Whitney U test was used in the analysis of independent quantitative data. The Wilcoxon test was used to study dependent quantitative data. The Statistical Package for Social Sciences version 27.0 was used in the statistical analysis.

Results

Ninety-two LSD patients were enrolled in this study. The patients' details are shown in Figure 1. The mean age of the patients was 22.5±16.6 years (min: 1.6, max: 70.0, median: 17.2). Fourty-seven (51.1%) of the patients were female and 45 (48.9%) were male. ERT was given to MPS I, MPS II, MPS IVA, MPS VI, and Pompe patients once a week and MPS VII, Gaucher, and Fabry patients every two weeks. In one year, 317 telemedicine interviews were performed (Table I).

Our study evaluated the percentage of ERT disruptions at quarterly intervals. The total skipped treatment rate within one year was 17.1%. The most frequent interruptions in treatments occurred in April-May-June, 2020 and October-November-December, 2020 with rates of 23.6% and 19.7%, respectively (Table I). The Turkish Government imposed social isolation in some periods in order to prevent any unfavorable effects of the pandemic. The periods of the most frequent treatment skipping months were consonant with these times of social isolation. When treatment compliance was evaluated according to gender, there was no significant difference between females and

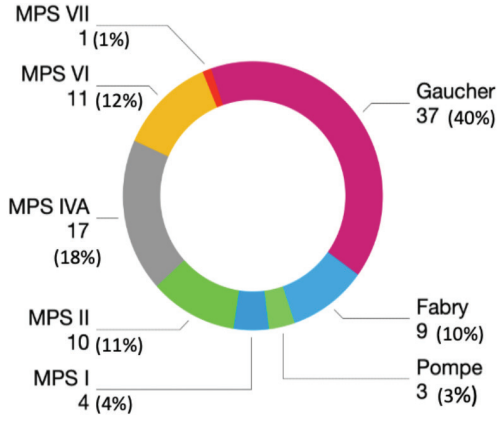

Figure 1. Distribution of the diseases

males with rates of 15.1% and 19.1%, respectively (p=0.814) (Table II).

Compliance with treatment was compared according to the age groups of under 18 years and over 18 years. The interruption of treatment in patients under 18 years was lower than patients over 18 years, with rates of 10.1% and 25.3%, respectively. A positive correlation was detected between the age of patients and the interruption periods of treatment (p=0.040) (Table II).

When the impact of disease groups was evaluated, the most frequent disruption of treatment was seen in the Gaucher group (22.1%). The second highest disruption of treatment rate was 18.8% in MPSIVA patients and the third highest rate was 17.4% in MPS VI patients. The high skipping rate of MPSIVA may be due to the three patients who did not receive any treatment for one year (Figure 2).

Due to the small number of individuals in the Fabry and Pompe groups, comparisons regarding missed treatment rates were made between MPS and Gaucher. There was no

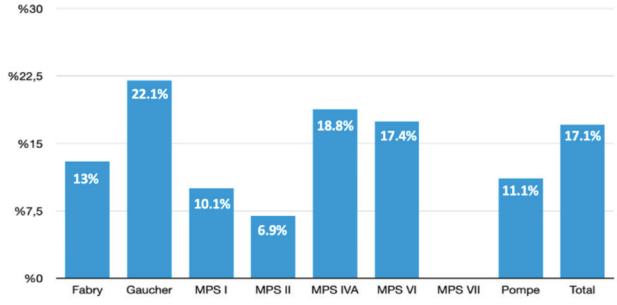

Figure 2. Treatment interruption percentages by patient groups

Table I. The number of telemedicine visits and percentage of skipped treatments by month

	Number of telemedicine visits (n)	Min.-Max.	Mean±SD
April-May-June 2020 Skipped treatment %	77	0.0-100.0	23.6±37.1
July-August-September 2020 Skipped treatment %	79	0.0-100.0	14.2±32.2
October-November-December 2020 Skipped treatment %	78	0.0-100.0	19.7±33.7
January-February-March 2021 Skipped treatment %	83	0.0-100.0	15.4±29.6
Total Skipped treatment %	317	0.0-100.0	17.1±26.3

Table II. Treatment skip percentages by gender

	Female (n=47)	**Male (n=45)**	**p-value**
	Mean±SD	**Mean±SD**	
April-May-June 2020 Skipped treatment %	24.8±35.8	22.4±38.7	p>0.05[m]
July-August-September 2020 Skipped treatment %	10.8±26.9	17.6±36.7	p>0.05[m]
October-November-December 2020 Skipped treatment %	17.6±30.3	21.9±37.0	p>0.05[m]
January-February-March 2021 Skipped treatment %	12.6±24.3	18.4±34.1	p>0.05[m]
Total Skipped treatment %	15.1±20.5	19.1±31.3	p>0.05[m]
	Age <18 (n=48)	**Age >18 (n=44)**	
	Mean±SD	**Mean±SD**	
April-May-June 2020 Skipped treatment %	16.9±31.9	31.6±41.4	p>0.05[m]
July-August-September 2020 Skipped treatment %	6.0±21.0	23.1±39.4	**p=0.017[m]**
October-November-December 2020 Skipped treatment %	10.3±23.5	30.0±39.9	**p=0.013[m]**
January-February-March 2021 Skipped treatment %	9.2±21.1	22.8±36.0	**p=0.048[m]**
Total Skipped treatment %	10.1±15.9	25.3±33.1	**p=0.040[m]**
	MPS (n=43)	**Gaucher (n=37)**	
	Mean±SD	**Mean±SD**	
April-May-June 2020 Skipped treatment %	22.8±35.8	26.5±40.6	p>0.05[m]
July-August-September 2020 Skipped treatment %	8.5±23.2	22.0±40.4	p>0.05[m]
October-November-December 2020 Skipped treatment %	14.1±25.9	29.6±40.8	p>0.05[m]
January-February-March 2021 Skipped treatment %	13.8±26.8	18.6±33.5	p>0.05[m]
Total Skipped treatment %	14.4±20.3	22.1±31.7	p>0.05[m]

[m]Mann-Whitney U test

significant difference in adherence to treatment between the MPS and Gaucher patients (p=0.813) (Table II).

Discussion

The COVID-19 infection was declared a pandemic in March, 2020 and it affected the whole world (1). Going to the hospital became challenging for patients with chronic illnesses during the pandemic. It has been reported that individuals with chronic diseases are more anxious and afraid of going to the hospital in a pandemic period (6). LSD are chronic diseases which result in the accumulation of toxic substances in the organs due to enzyme deficiency. Intravenous ERT is used to prevent this accumulation. Due to a fear of visiting hospitals during the pandemic, ERT was interrupted (11,12). Telemedicine is a good option for the ongoing management of chronic diseases and evaluating the patient's adherence to treatment and it has been widely used during the COVID-19 pandemic (4,13). However, increasing adherence to therapy with telemedicine may not always be successful. In our study, we evaluated the compliance to treatment of LSD patients using the telemedicine method over one year. To the best of our knowledge, this is the first long-term study in the literature to evaluate LSD patients' treatment compliance with telemedicine.

In our study, the percentage of missed treatment was 17.1% in the one year between April, 2020 and March, 2021. Kahraman et al. (11) showed by a questionnaire that 35 out of 75 LSD patients had missed treatment sessions. Their study was based on data from the first nine months of the pandemic.

The patient's fear of infection and uncertainty regarding the pandemic may have reduced the patients' visits to the hospital for treatment. In a survey study considering the effect of COVID-19 on patients with rare metabolic diseases, it was reported that almost half of the patients missed their ERT, and the most important reason for this interruption in treatment was the fear of going to the hospital and becoming infected (14).

Our study found that treatment skipping rates were lower in the patients in our study than in the literature. This indicates that telemedicine has a positive effect on adherence to treatment. Compliance with treatment was increased via repeated telemedicine in those patients with poor compliance.

Curfews were imposed at intermittent periods to prevent any unfavorable effects of the pandemic worldwide. There were curfews in Turkey in the months of March, April, May, June, October, and November in 2020. In the periods including these months, the rate of disruption in treatment was the highest (April-May-June was 23.6%, October-November-December was 19.7%). Due to curfews, it may be the case that patients do not come to the hospital because of anxiety. Those patients with chronic diseases were more anxious during the pandemic, and so avoided hospital visits (15-17). A decrease in treatment skipping rates was noted in the following months. The effect of telemedicine may have increased adherence to treatment. On the other hand, treatment disruption may have led to a lack of healthcare access in the months of government-enforced curfews and then improved in the other months due to improved access.

There was no significant difference between male and female patients regarding treatment adherence. The treatment disruption rate in patients under 18 years was lower than those over 18 years. This may be due to the fact that the parents take charge in compliance with the treatment for their children even during a pandemic. In other words, the fact that skipping treatment under the age of 18 is lower than that of adults may be associated with the higher observance of parents with their children's treatment.

Andrade-Campos et al. (12) reported the impact of the COVID-19 pandemic on Gaucher patients. They reported that 25% of the patients skipped treatment, which is similar to our study. MPS patients have severe comorbidities such as narrow airways and respiratory problems. Therefore, the risk of COVID-19 infection in MPS patients is expected to be higher compared to Gaucher patients (18). The treatment compliance of MPS and Gaucher patients was considered in terms of this situation. However, there was no significant difference between the two groups in our study.

Clinical worsening in LSD patients has been reported in the literature due to treatment discontinuation (19,20). In our study, the comorbidity experienced in patients whose ERT was disrupted was not evaluated. During the telemedicine interviews, we learned that a patient with MPS IVA deteriorated clinically. The 14-year-old male patient had a disruption in treatment of 25% during the pandemic period (March, 2020-September, 2020). He complained of an inability to walk during this period of disruption of treatment. The patient's 6-minute walking test was recorded at 325 meters in 2019. When the patient was evaluated, the diameter of the foramen magnum, which had been 10 cm in a cranial MRI performed in 2019, narrowed to 0.7 cm in September, 2020. The urinary glucosaminoglycan

level can level, which had been 78 mg/gr creatinine (<60) in 2019, increased to 135 mg/gr creatinine (<30) in September, 2020. It was observed that the clinical and laboratory findings were further exacerbated in this period when 25% of the treatment was interrupted for this patient.

Sechi et al. (8) reported that 49% of patients receiving ERT in hospitals experienced interruption, versus 6% of patients treated at home. In many European countries, home-based treatment is carried out under COVID-19 pandemic conditions for ERT compliance (21). Home-based ERT is not available in Turkey. Home-based ERT may be an option which can increase the compliance rates of LSD patients with treatment during the COVID-19 pandemic (19,22).

Study Limitations

There were some limitations in our study due to it being a single-center experience for only one year. Further studies with larger populations and long-term results may provide more data about ERT adherence with telemedicine.

Conclusion

In conclusion, telemedicine could be a good option for follow-up, management, and to ensure continuity of treatment of LSD patients in pandemic periods. Also, switching to home-based ERT may be an option which can increase treatment compliance for LSD individuals. The continuity of treatment of LSD patients is crucial in order to prevent comorbidities.

Ethics

Ethics Committee Approval: Ethics Committee approval was obtained from the Local Ethics Committee of Ege University (21-11.1T/43).

Informed Consent: All patients or their parents included in this study gave informed consent to take part in this study.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: M.Y.Ç., Concept: M.Y.Ç., H.Y., F.E., S.K.U., M.Ç., Design: M.Y.Ç., Data Collection or Processing: M.Y.Ç., Analysis or Interpretation: M.Y.Ç., E.C., Literature Search: M.Y.Ç., E.C., Writing: M.Y.Ç., E.C.

Conflict of Interest: No conflict of interest is declared by the authors.

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Case Report

A Neuroblastoma Case Presenting with Seizures Resistant to Anti-Epileptic Treatments

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ABSTRACT

Seizure is a rare symptom of paraneoplastic syndrome seen in neuroblastoma without a previous history. A 4-month-old male patient who was followed up with a preliminary diagnosis of an adrenal mass in pediatric oncology was admitted to hospital with a seizure. A diagnosis of undifferentiated neuroblastoma was made with a biopsy from an adrenal mass. Seizures were resistant to anti-epileptic therapy and they were completely under control with steroids on the 4th day of treatment. Electroencephalography (EEG) disturbances disappeared and no neurologic deficit was detected. This case, which presented with isolated seizure symptoms of neuroblastoma and was treated with steroids, was a very rare presentation in which symptoms and EEG disturbances disappeared. In neuroblastoma, autoimmunity may be involved in the pathogenesis of seizures, which is a rare finding of paraneoplastic syndrome and the option of immunotherapy should be considered.

Keywords: Epileptic seizure, neuroblastoma, autoantibody, steroid, paraneoplastic syndrome

Introduction

Neuroblastoma is a malignant neuroectodermal tumor and it is the most common extra-cranial solid tumor seen in childhood. Symptoms and signs are variable, depending on the localization of the tumor, metastasis, and paraneoplastic association (1). Seizure is rare as a first symptom. Paraneoplastic neurological syndromes in neuroblastoma may be associated with autoimmune epilepsy (OE) or opsoclonus-myoclonus-ataxia syndrome (OMAS). OE, in which acquired immunity plays a role, exists in the etiology of seizures. OMAS is a clinical syndrome consisting of involuntary chaotic eye movements, myoclonus of the extremities, and ataxia (1-3). As information on the role of autoimmunity and neuroinflammation in epileptogenesis has increased, immunotherapy options have begun to be offered and seizure control has begun in some patients (3,4). Although it has been discussed as to whether it is a paraneoplastic symptom or not, seizure is rare. We present a 4-month-old patient with neuroblastoma who did not respond to anti-epileptics, and whose seizure control was achieved with immunotherapy.

Case Report

A 4-month-old boy who had no previous history of seizures was referred to our center from another center with generalized clonic seizures occurring 3 times within 24 hours. The patient's neurological development and examination were normal. Among the laboratory results obtained, hemogram, liver and kidney function tests, glucose, electrolytes, and ammonia values were normal. No feature was found in cerebrospinal fluid findings. In EEG, slow waves with a generalized amplitude of 300-350 mV, showing the highest amplitude in the left occipital region, were observed at frequent intervals. The patient, who had a history of antenatal hydronephrosis, was followed up in pediatric oncology after a mass was noticed in the left surreal lobe in postnatal abdominal ultrasonography (USG). There was no infection or drug exposure in the antenatal and postnatal periods. Contrast-enhanced abdominal magnetic resonance imaging (MRI) revealed a solid mass of 5x5x5 cm in size (Figure 1). In the follow-up, a biopsy was performed

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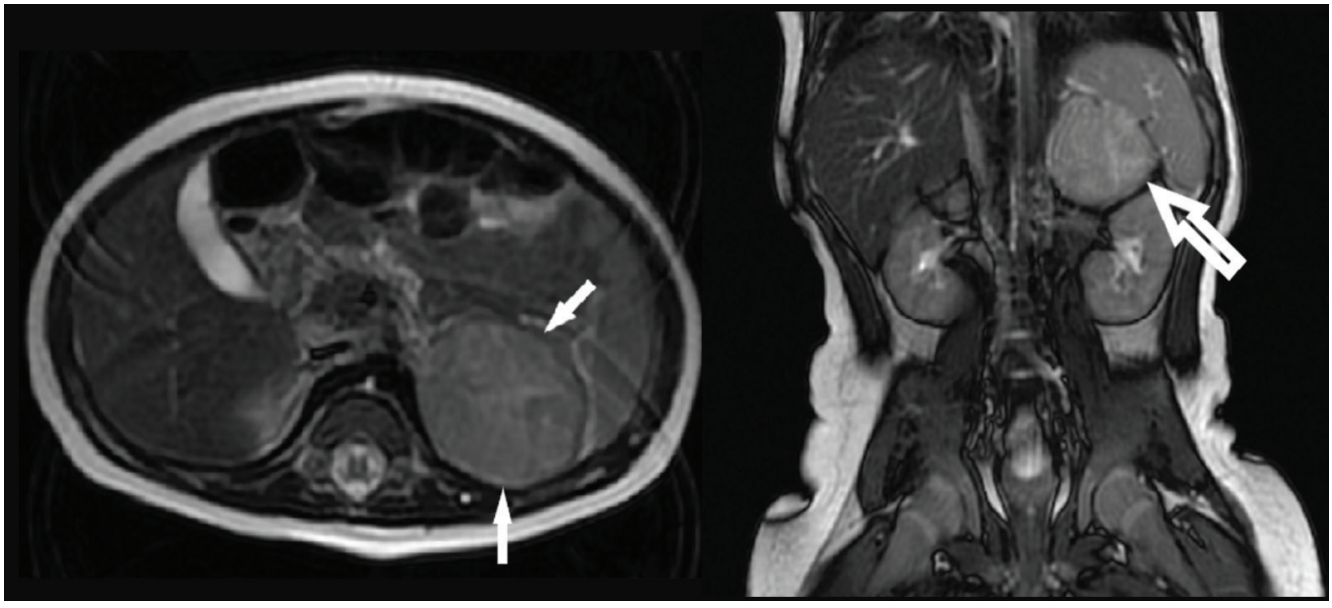

Figure 1. A homogeneous, space-occupying formation with intense contrast enhancement approximately 5x5x5 in size, in the left adrenal lobe

on the patient as an increase in the size of the mass was detected on USG and an increase in the urinary catecholamine level was observed. The biopsy of the patient's pathology resulted in a very low-risk undifferentiated neuroblastoma. The patient's age was below 18 months, n-myc amplification and 11q23 loss were negative in molecular examination, the tumor was non-metastatic and after total resection (when he was 5 months old) due to the absence of macroscopic and microscopic residues, the patient was classified as 'Very Low-Risk Group' and chemotherapy was not given to the patient.

Anti-epileptic treatment of levetiracetam, phenytoin, and topiramate was administered to the patient. In the follow-up of the patient, seizures continued intermittently despite triple anti-epileptic therapy. It was thought that paraneoplastic syndrome might play a role in the pathogenesis of the seizures, and steroid treatment was started (dexamethasone 0.4 mg/kg/day for 4 weeks). On the 4th day of steroid treatment (in the fourth month of the patient), seizures stopped and the anti-epileptic drugs, except for levetiracetam, were discontinued. Intravenous gammaglobulin was not given. Pathologies previously detected in control EEGs disappeared. Brain MRI taken for etiopathogenesis was normal. Our patient's antibody prevalence in epilepsy (APE) score was 4. The studied paraneoplastic autoantibody panel (Anti-Ri, anti-Yo, anti-Hu, anti-CV2, anti-Amphiphysin, anti-Ma2/Ta, anti-Recoverin, SOX1 antibody, Zic4, GAD65, Tr/DNER) was negative. Our patient's 'response to immunotherapy in epilepsy' (RITE) score was 5. No pathology was found in the gene panel for epilepsies of genetic origin, which is common in the infantile period. In our case with neuroblastoma, opsoclonus-myoclonus, which is a sign of paraneoplastic syndrome, was not observed either in the first application or in the 3-month follow-up. The patient was followed up by the pediatric neurology and oncology clinics and was seen to be completely healthy.

Discussion

OE, in which acquired immunity plays a role, exists in the etiology of seizures. A significant proportion of cryptogenic epilepsies have been attributed to autoimmunity, or a possible autoimmune cause (3,5,6). Discussion has started as to whether the seizures, which are rarely reported in neuroblastoma cases, are accidental or autoimmune (as a part of the paraneoplastic syndrome). OE may be isolated or be a part of the paraneoplastic syndrome (5-7).

A recent, prospective study reported serologic findings among consecutively evaluated patients presenting with epilepsy of unknown etiology. The same study also evaluated a scoring system known as the APE score as a model to predict the detection of these Abs based on the patients' clinical presentation and initial neurologic evaluation. The score was prospectively assigned to all enrolled patients before Ab testing. An APE score of ≥4 had a sensitivity and specificity of 82.6% and

82.0%, respectively (7-10). In that study, patients who received immunotherapy, autonomic dysfunction, faciobrachial dystonic seizures/oral dyskinesia, early initiation of immunotherapy, or who had the presence of antibodies targeting plasma membrane proteins (cell-surface antigens) were associated with favorable seizure outcomes. The sensitivity and specificity of an RITE score ≥7 to predict favorable seizure outcomes were 87.5% and 83.8%, respectively (7-14).

In OMAS, which is a paraneoplastic syndrome seen in neuroblastoma, movement disorders such as opsoclonus-myoclonus are detected, but seizures are not included in its definition. Among these, seizures with antibody (anti-Hu) positive OMAS were reported in only two cases. A 20-month-old infant with Turner syndrome presented with abdominal neuroblastoma and OMAS developed progressive hearing loss and seizures despite the complete removal of the tumor. Due to the disappearance of opsoclonus-myoclonus and the absence of new neurological symptoms with intravenous immunoglobulin therapy, the authors suggested that they provide direct support for the autoimmune basis of paraneoplastic symptoms associated with neuroblastoma (1,2,15). Another 11-year-old patient with anti-Hu (+) neuroblastoma first presented with epilepsy partialis continua (EPC) and later developed OMAS (16).

Although anti-Hu (+) encephalomyelitis cases have mostly been reported in association with small cell lung cancer in adulthood, pediatric encephalomyelitis cases are rarely seen (17,18). Anti-Hu (+) encephalomyelitis cases may progress as resistant epilepsy or 'Epilepsia partialis continua' in the follow-up (19,20). Only two cases of limbic encephalitis associated with neuroblastoma have been reported, and neither of these had prior OMAS (21,22). In both cases, limbic encephalitis preceded the diagnosis of the tumor and was associated with anti-Hu antibodies. Neurologic symptoms can precede the diagnosis of the neoplasm.

There are also case reports of neuroblastoma presenting with seizures without OMAS. White et al. (23) reported seizure and developmental delay in two cases without OMAS. The first case presented with infantile spasm and was diagnosed with neuroblastoma at the age of 5. In the second case, neuroblastoma was detected when the female patient was 4 weeks old, she had presented with neonatal seizure when she was 1-day old. In both cases, epilepsy resistance to antiepileptic treatments and significant growth retardation developed. The authors suggested that in these cases, epilepsy resistance to antiepileptic treatments and growth retardation may be coincidental or immune mechanisms may play a role in their pathogenesis (23).

Our case presented with seizures which did not respond to antiepileptic therapy. It was reported that he was followed up for a mass compatible with adrenal neuroblastoma in the postnatal abdominal USG. For this reason, IV dexamethasone was initiated, considering that it might be a symptom of paraneoplastic syndrome, while further investigations for seizures were performed. All seizures disappeared 4 days after steroid treatment was initiated and antiepileptic drugs were discontinued.

Paraneoplastic syndrome in neuroblastoma is most common in the age range of 18-24 months and is not expected for less than 6 months due to immune system development (24). As in our case, a case with pelvic neuroblastoma who presented with their first seizure complaint has been reported in the literature (24,25). The authors suggested that the seizure may be part of the non-classical paraneoplastic syndrome. Neuroinflammation both in the innate and acquired immune system, which plays a role in pathogenesis and epileptogenesis, is subtle and immunotherapy should be discussed (3). In our case, seizure control, improvement in EEG, and normal neurological development after steroid treatment suggest autoimmune and/or neurotransmitter-mediated paraneoplastic syndrome. Although it is thought that autoimmunity plays a lesser role when the age of the patient was taken into consideration, the disappearance of all symptoms with steroids cannot rule out the role of autoimmunity in neuroblastoma. In addition, the release of neurotransmitters, which are secreted from the tumoral tissue and play a role in epileptogenesis, may additionally contribute to the occurrence of seizures (20,26).

We present a rare case of refractory seizures and neuroblastoma with good outcomes after treatment. It would be useful to consider this relationship when evaluating seizures of unknown origin in the first years of life. Thus, in similar cases, immune-modulatory therapy may be considered primarily due to possible autoimmune etiopathogenesis.

Ethics

Informed Consent: Informed consent was obtained from the patient's parents.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Concept: Ç.Ç.K., Design: Ç.Ç.K., A.K.A., C.Y., Data Collection and/or Processing: Ç.Ç.K., Analysis and/or Interpretation: Ç.Ç.K., Literature Search: S.A.O., M.P., Ç.Ç.K., Writing: Ç.Ç.K., M.P.

Conflict of Interest: The authors declared that there were no conflicts of interest.

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Case Report

Distinctively Different Phenotypes of Two Cases with a Rare Karyotype of 45,X/47,XYY Mosaicism: Case Report and Literature Review

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ABSTRACT

The 45,X/47,XYY mosaicism is an extremely rare genetic disorder with highly phenotypic manifestations such as ovotesticular disorders of sexual development, mixed gonadal dysgenesis and Turner syndrome. Herein, we report two cases with very distinctive phenotypes despite having the same sex chromosome mosaicism of 45,X/47,XYY. It should be kept in mind that the rare type of sex chromosome mosaicism of 45,X/47,XYY may present with genital phenotypes ranging from normal female to male characteristics.

Keywords: 45,X/47,XYY, Turner syndrome, ambiguous genitalia, gonadal dysgenesis

Introduction

The 45,X/47,XYY mosaicism is a rare chromosomal anomaly resulting from postzygotic mitotic non-disjunction. The 45,X/47,XYY karyotype is a rare cause of disorders of sexual development (DSD) and presents with highly variable phenotypic features (1). Short stature has been reported in most of the cases with/without Turner stigmata such as webbed neck, horseshoe kidney and cubitus valgus (2). The characteristic features of this disorder remain unclear because of its low incidence (1). We aimed to present two cases with distinctly different phenotypes from each other despite having the same sex chromosome mosaicism of 45,X/47,XYY.

Case Report

Case 1

A male aged 14 years and 4 months presented to our outpatient clinic due to short stature and hypospadias. He was born as the first child of healthy non-consanguineous parents and delivered at term by normal vaginal delivery after an uneventful pregnancy. His family history was unremarkable. His past medical history revealed that he had been operated on two times for penoscrotal hypospadias when he was one and five years old. In the second session, gonadectomy was performed for atrophic left testis and he lost this during follow-up thereafter.

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On physical examination at presentation, his weight was 41 kg [-1.49 standard deviation score (SDS)], and his height was 150.3 cm (-1.93 SDS). His Tanner staging was axillary hair (+), pubic hair stage 3 and the right testis was palpable with a volume of 12 mL in the scrotum. However, the left testis was not palpable in the scrotum or inguinal region. The stretched penile length was 7.2 cm and proximal penile hypospadias was observed. Other systemic physical examination features were unremarkable. No Turner's stigmata were visualized.

The test results of his hormonal evaluation were as follows; follicle stimulating hormone (FSH): 9.37 mIU/mL (N, 1.2-10.3), luteinizing hormone (LH): 1.16 mIU/mL (N, 0.2-5), testosterone: 162.25 ng/dL (N, 100-1,200), AMH: 2.16 ng/mL (N, 2-30,7 ng/mL), adrenocorticotropic hormone: 32 pg/mL (N, 7-6,999) and cortisol: 11.6 µg/dL (N, 2-25). Mid-parental height was 164 cm (-1.98 SDS) and bone age was consistent with his chronological age. On hormonal examination for short stature, insulin-like growth factor-1 was 260 ng/mL (-1 SDS), insulin-like growth factor binding protein-3 was 6,270 ng/mL (+0.18 SDS) while peak growth hormone release with L-dopa was within normal limits (15.5 ng/mL). Ultrasonographic examination of the scrotum revealed that the right testis was in scrotum. However, the left testis was not seen in the scrotum, inguinal or pelvic region and also no Mullerian structure was detected on pelvic ultrasonography. Chromosome analysis from peripheral blood cells revealed the presence of 45,X/47,XYY mosaicism (46-44, respectively) (Figure 1). The case was discussed in DSD council and hypospadias repair was performed. On his last physical examination when he was 17 years and 10 months of age, his weight was 51.5 kg (-2.4 SDS), his height was 156 cm (-3.1 SDS), his body mass index was 20.9 kg/m^2 (-0.7 SDS), the right testis was palpable as 20 mL in the scrotum, the left testis was non-palpable, pubic hair development was Tanner stage 5, and stretched penis length was 10.4 cm. Laboratory examination showed FSH: 19.3 mIU/mL (1.2-10.3 mIU/mL), LH: 11.1 mIU/mL (0.2-5 mIU/mL), and total testosterone: 716 ng/dL. He described normal erection and ejaculation. Spermiogram analysis revealed 1.2 million/mL sperm, which suggests insufficiency and therefore Kruger assessment could not be carried out. He was monitored for tumour markers including Alpha-Fetoprotein and beta-human chorionic gonadotropin and testicular ultrasound for the probable development of gonadal tumours and fortunately, no evidence of gonadal tumour had been detected at the time of writing. Chromosome analyses of the parents were unremarkable. Written informed consent was obtained from the patient and his parents for the publication of this case report and any accompanying images.

Case 2

A female aged 16 years and 9 months presented to our outpatient clinic for primary amenorrhea. She was the first child of non-consanguineous parents and delivered at term (40-weeks) by normal vaginal delivery with a weight of 2,100 gr. Her family history was unremarkable, but pregnancy was achieved by ovarian stimulation therapy.

On physical examination, her weight was 46.8 kg (-1.67 SDS), her height was 149.6 cm (-2.23 SDS), blood pressure was 110/66 mmHg, and webbed neck, clinodactyly, low-set ears, cubitis valgus, low hairline and multiple pigmented nevus were observed. Pubertal Tanner stage was compatible with

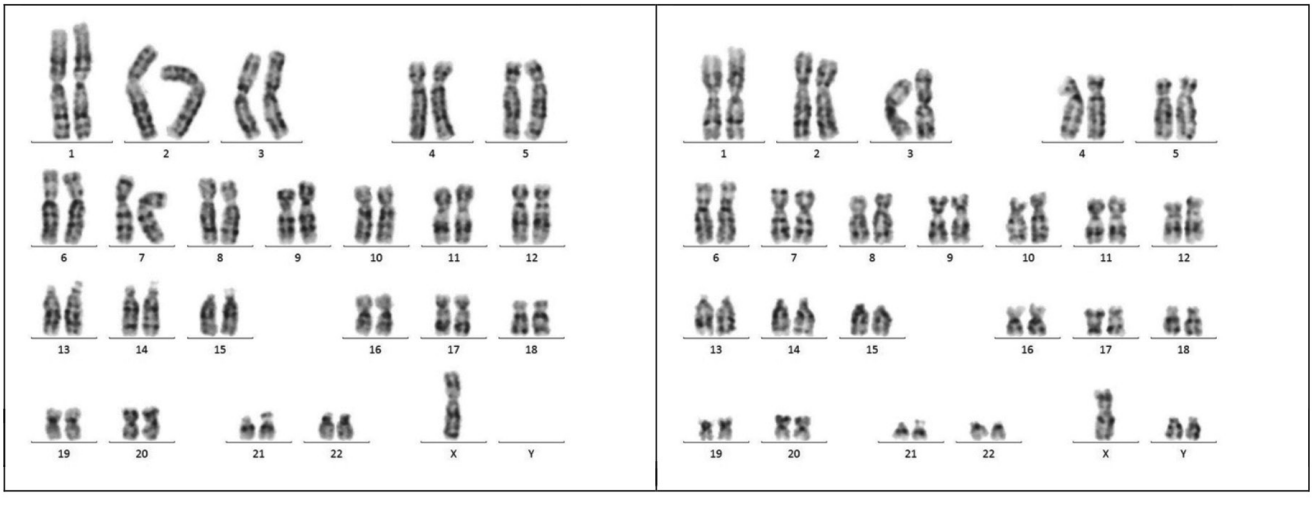

Figure 1. Karyotype of case 1

stage 1 for breast development and stage 5 for pubic hair. No enlarged clitoris or palpable gonads were visualized (Figure 2A, 2B and 2C). Mid-parental height was 167.5 cm (+0.35 SDS) and bone age was compatible with 12 years of age according to the Greulich and Pyle atlas. Initial laboratory investigations showed normal full blood count, electrolytes and renal, liver, thyroid functions. Hormone analysis revealed hypergonadotropic hypogonadism as follows; FSH: 138.4 mIU/mL LH: 29.3 mIU/mL and estradiol <10 ng/dL. Her echocardiography was normal. An ultrasonographic examination of the pelvis revealed atrophic uterus and streak gonads located in their normal positions. Chromosome analysis from peripheral blood cells revealed 45,X/47,XYY (15-35, respectively) (Figure 2D). Bilateral gonadectomy was performed and histopathological examination revealed streak gonadal structure and no evidence of malignancy. This patient, who presented with Turner syndrome phenotype, was raised as a girl and oestrogen replacement therapy was started. Growth hormone replacement therapy was not initiated due to the family's disapproval. Written informed consent was obtained from the patient and her parent for publication of this case report and any accompanying images.

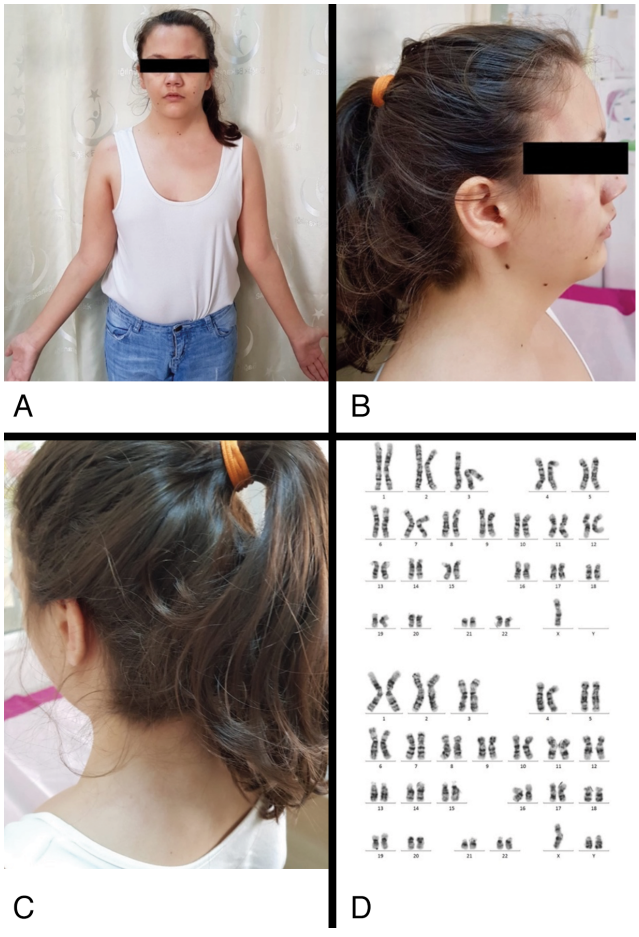

Figure 2. The phenotypic features of case 2 (A, B, C) karyotype of the case 2 (D)

Discussion

We have reported two 45,X/47,XYY mosaicism cases showing different phenotypic features and reviewed the literature based on these cases. Chromosomal mosaicism is generally considered to be the consequence of an event occurring at an early stage of cell division. The 45,X/47,XYY mosaicism is a quite rare cause of sex chromosome DSD. Although its incidence is not clearly known, the incidence of 45,X/46, XY and 45,X/47,XYY chromosomal mosaicism is given as 1.7/10,000 and cases with the 45,X/47, XYY karyotype constitute a small part of this group (1).

Patients with 45,X/47,XYY were included in the DSD group due to sex chromosome disorder in the 2006 DSD Consensus and termed as "mixed gonadal dysgenesis" (3).

Phenotypic manifestations are highly variable in the 45,X/47,XYY karyotype. Minor or major phenotypic differences can be seen in individuals with the same mosaic karyotype. While some of the male cases have ambiguous genitalia; in some cases, fertility problems may be observed with completely normal genitalia (2). Some cases may present with Turner stigmata with female phenotype. The first case with the 45,X/47,XYY karyotype was reported in 1961 by Jacobs (4). This case had female genitalia and Turner stigmata such as short stature and webbed neck, similar to our case number 2. The underlying mechanism of the correlation between phenotype and mosaicism is not yet clear, which may be related to the very low incidence of this mosaicism (2). When all previously reported cases in the literature were reviewed, it was found that the mosaicism ratio did not affect the phenotype. Further cases are required to clarify this issue. In 1991, Pettenati et al. (5) reported on the largest series to date and focused on the clinical discrepancy of those cases diagnosed with prenatal or postnatal features. A total of 18 cases with heterogeneous phenotype having the 45,X/47,XYY karyotype have been reported to date. The median age of the previously reported cases was 11 years (interquartile range: 0-20). Four out of these 18 cases were diagnosed prenatally. Short stature was reported in 7 cases (38.9%), and the characteristics of Turner syndromes were reported in 6 (5 of them were raised as female, and the remaining one as male) patients. Five had ambiguous genitalia (2 hypospadias, 2 enlarged clitorises and one patient had small phallus, hypospadias and fused labioscrotal folds). Nine cases were raised as

female (56.3%) and 7 cases as male (43.7%). The detailed clinical and laboratory characteristics of the literature cases are presented in Table I.

The estimated risk of developing gonadoblastoma vary from 7% to 30% in female phenotype gonadal dysgenesis with 45,X mosaicism containing Y chromosome and in some studies bilateral prophylactic gonadectomy is recommended (6,7). However, in some studies, a very low incidence of gonadoblastoma development has been reported. Although prophylactic gonadectomy in the female phenotype is still controversial, it seems to be the only way to exclude malignancy (8). Prophylactic gonadectomy was applied in our case number 2, who was raised as a girl and, fortunately, no histopathological evidence suggesting gonadoblastoma was detected. It is unclear whether or not to remove the testes in phenotypic normal males. Close follow-up of the gonads with physical examination is the best prevention against the development of gonadoblastoma and dysgerminoma (5). In our first case, the atrophic testis was removed and the intact testis was preserved. In the follow-up, he was monitored for some tumour markers and testicular ultrasound for the potential development of gonadal tumours.

In 2020, Zhang et al. (9) reported pregnancy, and normal vaginal delivery resulting from in vitro fertilization using spermatozoa surgically retrieved from a male patient with 45,X/46,XY mosaicism. Similarly, in patients with male phenotype having 45,X/47,XYY chromosomal mosaicism, it may be possible to achieve fertility if the gonads are preserved.

An insufficient number of spermatozoa were observed in the semen analysis of our case raised as male, and he will be re-evaluated when fertility is planned.

Conclusion

In this study, we presented two cases of 45,X/47,XYY mosaicism with distinctly different phenotypes. Since the characteristics of this disorder remain unclear because of its low incidence, further studies are required to clarify this issue. It should be kept in mind that the rare type of sex chromosome mosaicism of 45,X/47,XYY may present with genital phenotypes having normal female or male characteristics.

Table I. Clinical characteristics of patients with 45,X/47,XYY karyotype

Case no	Reference	Age at assessment	Clinical features	Reared gender	FSH/LH (mIU/mL)	Karyotype percent		External genitalia	Internal genitalia	Gonads
						45,X %	47,XYY%			
1	Jacobs (4)	20 y	Short stature Neck webbing	F	NA	69	25	Normal female with sexual hypoplasia	No laparotomy	No laparotomy
2	Cooper et al. (10)	16 y	Short stature Shield chest Widely spaced nipples	F	NA	2	96	Normal female with sexual hypoplasia	Absent uterus Blind Fallopian tubes	Streak gonads in position of ovaries
3	Trowell and Hamilton et al. (5,11)	29 y	Short stature	M		63	37	Normal male	NA	Testicular tissue; tubules lined only with Sertoli cells, well-preserved Leydig cells
4	Mulcahy et al. (11)	NB	Enlarged clitoris Bilateral inguinal hernia	F	NA	60	40	Enlarged clitoris	Normal female	Gonads attached to broad ligament; testicular tissue
5	Lisker et al. (12)	22 y	Short stature High arched palate Low-set ears Low hairline Short neck Cubitus valgus Multiple pigmented nevus	F	58/130	53	47	Normal female	Streak gonads Ovarian stroma	
6	Pettenati et al. (5)	Fetus	None	M	NA	25	75	Normal male	Normal male	
7	Pettenati et al. (5)	Fetus	None	M	NA	90	10	Normal male	No laparotomy	No laparotomy
8	Pettenati et al. (5)	6 m	No abnormalities except genitalia	F	NA	83	17	Enlarged clitoris	Infantile uterus Small Fallopian tubes and cervix Infantile vagina	Left inguinal testis Right streak gonad
9	Pettenati et al. (5)	11 y	Short stature Shield chest Low hair line Cubitus valgus Pigmented nevi	F	NA	10	90	Normal female	Partially uncanalised uterus	Streak gonads No evidence of malignancy

Table I. Continued

Case no	Reference	Age at assessment	Clinical features	Reared gender	FSH/LH (mIU/mL)	Karyotype percent		External genitalia	Internal genitalia	Gonads
						45,X %	47,XYY%			
10	Pettenati et al. (5)	16 m	Short stature Epicanthal folds Depressed nasal bridge Systolic murmur	M	NA	18	82	Midshaft penile hypospadias	Presence of mullerian duct remnants and rudimentary vagina and uterus	NA
11	Pettenati et al. (5)	Fetus	No abnormalities except genitalia	Terminated	NA	20	80	Normal male	No Mullerian structure	Undescended testes (autopsy)
12	Pettenati et al. (5)	NB (10-day old)	High arched palate Posteriorly rotated ears Micrognathia Neck webbing Pectus excavatum	M	NA	10	90	Small phallus Severe chordee Fused labioscrotal folds Undescended testes	Uterus with left Fallopian tube, rudimentary right Fallopian tube and vagina	Immature testes
12	Fukui et al. (8)	NA	Short stature High arched palate Cubitis valgus	F	10.9/0.2 (50.2/9.0)*	12	87	Normal female	Hypoplastic uterus Normal vagina	Ovarian stroma with nests of gonadoblastoma
14	Lin et al. (13)	Fetus	Hypoplastic nasal bone Large facial angle	Terminated	NA	66	34	Normal male	NA	
15	Anık et al. (14)	NB	No abnormalities except genitalia	M	3.9	80	20	Hypospadias Posteriorly fused labia majora	No Mullerian structure	NA
16	Farrugia et al. (1)	NA	None	M	NA	NA	NA	NA	NA	NA
17	Farrugia et al. (1)	15 y	High arched palate Skin pigmentation Long fingers Deafness Reduced vision TAPVD	F	NA	NA	NA	NA		PLAP (+) gonads
18	Farrugia et al. (1)	NA	None	F	NA	NA	NA	NA	NA	

Table I. Continued

Case no	Reference	Age at assessment	Clinical features	Reared gender	FSH/LH (mIU/mL)	Karyotype percent		External genitalia	Internal genitalia	Gonads
						45,X %	47,XYY%			
Present case 1		14 y	Short stature	M	9.3/1.1	46	44	Hypospadias	Undescended testes	One atrophyic testis
Present case 2		16 y	Short stature Neck webbing Clinodactyly Low hair line Cubitis valgus Pigmented nevi	F	138.4/29.3	30	70	Normal female	Hypoplastic uterus	Streak gonads

*Following luteinising hormone-releasing hormone stimulating test. Normal references of the parametres as follow: FSH: prepubertal: 0.2-11.1 mIU/mL Tanner stage 2: 1.7-4.6 mIU/mL Tanner stage 3: 2.5-7.04 mIU/mL Tanner stage 4: 1.2-7.3 mIU/mL Tanner stage 5: 1.02-9.2 mIU/mL, LH prepubertal <0.2 mIU/mL Tanner stage 2: 0.2-4.9 mIU/mL Tanner Stage 3: 0.2-5 mIU/mL Tanner stage 4-5: 0.4-7 mIU/mL.
y: Year, m: month, NB: Newborn, M: Male, F: Female, FSH: Follicle stimulating hormone, LH: Luteinizing hormone, NA: Not available, TAPVD: Total anomalous pulmonary venous drainage, PLAP: Placental-like alkaline phosphatase

Ethics

Informed Consent: Written informed consent was obtained from the patient and his parents for the publication of this case report and any accompanying images.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Ö.K., S.A., Ö.N., Concept: Ö.K., S.A., Beh.Ö., Design: Ö.K., S.A., Data Collection or Processing: T.K., G.A., B.Ö., Analysis or Interpretation: Ö.K., K.M.E., Y.B.K., Literature Search: Ö.K., S.A., Writing: Ö.K., S.A., K.M.E., Beh.Ö.

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