Year: December 2024 Volume: 11 Issue: 4

E-ISSN: 2587-2478

The Journal of Pediatric Research

JPR
The Journal of Pediatric Research
Official Journal of Ege University Children's Hospital

EDITORIAL BOARD

Volume: 11, Issue: 4, December 2024

The Journal of Pediatric Research is the official publication of Ege University Faculty of Medicine, Department of Pediatrics and Ege Children's Foundation.

Publisher Contact
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34093 İstanbul, Turkey
Phone: +90 (530) 177 30 97 / +90 (539) 307 32 03
E-mail: info@galenos.com.tr/yayin@galenos.com.tr
Web: www.galenos.com.tr
Publisher Certificate Number: 14521

Online Publication Date: December 2024
E-ISSN: 2587-2478
International scientific journal published quarterly.

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EDITORIAL

Dear JPR Readers,

We are proud and happy to announce that the forth issue of "The Journal of Pediatric Research" in 2024 has been published. In this issue, we present to you 10 articles, including 8 original pieces of research and 2 case reports.

The World Children's Day is celebrated on the 20th of November to commemorate the Declaration of the Rights of the Child by the UN General Assembly on the 20th of November 1959. The United Nations Convention on the Rights of the Child is an important agreement by countries which have promised to protect children's rights. The Convention on the Rights of the Child explains who children are, all of their rights, and the responsibilities of governments. All the rights are connected, they are all equally important and they cannot be taken away from children.

Children's rights are human rights. They are non-negotiable and universal. However, in too many places today, children's rights are being misunderstood, disregarded or even denied and attacked. Upholding children's rights is the compass to a better world - today, tomorrow and into the future.

I would like to acknowledge the authors, the reviewers, the 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.

I wish all the children of the World a healthy, happy, safe and peaceful new year.

Kind Regards,
Prof. Dr. Zülal Ülger Tutar

J Pediatr Res 2024;11(4):190-7
DOI: 10.4274/jpr.galenos.2024.90023

Original Article

The Effects and Mechanisms of Sucralfate on Intestinal Epithelial Cells in an *In Vitro* Model of Necrotizing Enterocolitis

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ABSTRACT

Aim: Necrotizing enterocolitis (NEC) is an important disease which particularly affects premature babies. Although its pathogenesis is not fully explained, it is thought that the mucosal barrier of the intestine is disrupted. Sucralfate is a cytoprotective drug which supports the mucosal barrier. This study aimed to investigate the effects and mechanisms of sucralfate on enterocytes in an *in vitro* NEC model.

Materials and Methods: Intestinal epithelial cells were cultured. NEC was created with lipopolysaccharide (LPS). Sucralfate dose and concentration (106 µL/cm^2 2:1 diluted) were determined according to the results of the cell viability test. Control group: This was the cell culture without treatment. Sham group: Only sucralfate was applied to the cell culture. NEC group (NG): Only LPS was applied to the cell culture. Treatment group (TG): the cell culture was first treated with LPS and then sucralfate. Prophylaxis group (PG): First sucralfate and then LPS were applied to the cell culture. Terminal deoxynucleotidyl transferase dUTP end-labeling (TUNEL), caspase 3-8-9, RIPK 1-3, MLKL, occludin, claudin, ICAM and MadCAM were investigated immunohistochemically. Differences between the groups were compared via the one-way ANOVA test.

Results: TNF-α and IL-8 levels were higher in the NG ($p<0.05$). TUNEL positive cells were 65.6±8.2% in the NG and 15.4±3.2% in the TG ($p<0.05$). Caspase-8,9 and RIPK1 were higher in the NG ($p<0.05$). The RIPK3 level was low in the NG ($p<0.05$). MLKL was high in the NG, low in the TG and PG ($p<0.05$). ICAM-1 was not significantly different between groups. MadCAM-1 was higher in the NG than in the TG and PG ($p<0.05$). Occludin expression was high and claudin expression was low in the TG ($p<0.05$).

Conclusion: *In the vitro* NEC model, apoptosis and necroptosis and the expression of cell adhesion molecules change. Sucralfate helps regulate apoptotic - necroptotic activity and cell adhesion molecules. The prophylactic administration of sucralfate does not appear to be as effective as therapeutic administration.

Keywords: Necrotizing enterocolitis, sucralfate, cell culture, apoptosis, necroptosis, cell adhesion molecules

Introduction

Necrotizing enterocolitis (NEC) primarily affects premature infants and it is associated with significant morbidity and mortality. While its pathogenesis is not fully understood, NEC is characterized by a disruption of the intestinal mucosal barrier and an invasion of the intestinal wall by Gram-negative bacteria (1). Despite ongoing research, no definitive solution has been found, though breast milk and some probiotics may be effective in preventing NEC.

Sucralfate (aluminum sucrose sulfate) is a cytoprotective agent which supports the mucosal barrier. Approved

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Received: 03.07.2024 **Accepted:** 22.09.2024

by the Food and Drug Administration for preventing or treating upper gastrointestinal ulcers, sucralfate adheres to inflamed epithelium and has shown effectiveness in epithelial wound healing, chemotherapy-induced mucositis, radiation proctitis, oral ulcers, and burn wound treatment (2,3). Given that NEC involves mucosal barrier disruption leading to inflammation and necrosis, sucralfate may be beneficial in treating or preventing NEC. Previous studies demonstrated that oral sucralfate partially prevented and treated NEC in a neonatal rat model and proved beneficial in preventing ischemia-reperfusion injury in another model (4,5). However, these studies did not clarify the extent of sucralfate's reach to the damaged bowel area or its underlying mechanism of action.

This study aimed to investigate the effects and mechanisms of sucralfate, which forms a protective layer on damaged intestinal epithelium, in an *in vitro* experimental NEC model.

Materials and Methods

This study's design was approved by the Health Sciences Ethics Committee of Manisa Celal Bayar University Faculty of Medicine (date: 02.06.2021, approval no.: 20.478.486/842) and it was funded by Manisa Celal Bayar University Scientific Research Projects Office (2021-073).

Enterocyte Cell Culture

An Intestinal Epithelioid Cell line (IEC-6, CRL-1592, ATCC, USA) was purchased from ATTC. The cells were cultured with 90% fetal bovine serum, 1% penicillin-streptomycin and 10% α-MEM (Minimum Eagle's Medium) at 37 °C, 5% CO_2 until 80% confluency.

NEC Model

Lipopolysaccharide (LPS, L2630, Sigma-Aldrich, Germany, 200 µg/mL) was added onto IEC-6 cells (6,7) for 24 and 48 hours to model. After this, the levels of TNF-α and IL-8 in the culture media were determined by ELISA (DZE201120083 for TNF-α, and DZE SRB-T-83151 for IL-8, Sun Red Biotechnology Company) using the instructions of the kits' protocols. Samples were read in a microplate reader at 450 nm absorbance.

Cell Viability Test (MTT) at Different Sucralfate Doses and Concentrations

The amount of sucralfate (Antepsin 250 mL suspension, Bilim Pharmaceuticals, Turkey) to be applied to the cell culture wells was calculated as follows: Based on the adult body surface area and the maximum daily dose of sucralfate of 4 g, the maximum dose that could be applied per square centimeter was calculated to be 108 µL/cm^2. In determining the optimal nontoxic dose, values below (104 and 106 µL/cm^2) and above (112-114 µL/cm^2) the calculated dose were also tested.

Evaluation of the cytotoxicity of sucralfate on IEC-6 cells was performed using the colorimetric method, 3'-(4,5-Dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) salt. Cells were seeded in 96-well culture (1x10^3 cells in each well) dishes with 100 µL of culture medium in each well and incubated for 24 hours. Doses of sucralfate were applied as 3 replicates and incubated for 24 or 48 hours. After incubation, 10 µL of MTT solution was added to each and incubated for 4 hours. The medium was removed and 50 µL of dimethyl sulfoxide was added and the absorbance was measured at 540 nm.

Experimental Groups

In the control group, the cells were cultured only in the culture medium. When the sucralfate was applied, the group was called Sham. In the NEC group, LPS was applied to the cell culture for 48 hours. After LPS administration, when the sucralfate was added for 48 hours, the group was called treatment. In the prophylaxis group, LPS administration was applied after sucralfate administration.

Terminal Deoxynucleotidyl Transferase dUTP End-labeling-TUNEL Method

Apoptotic cells were determined using the terminal deoxynucleotidyl transferase dUTP end-labeling (TUNEL) assay (S7101, Millipore, USA). All groups of cells were fixed with 4% paraformaldehyde for 30 minutes. After washing with phosphate buffer saline (PBS), they were permeabilized with 0.1% Triton X-100 for 10 minutes on ice and then washed with PBS. After incubation with Tdt enzyme at 37 °C for 1 hour, the samples were washed with equilibrium buffer solution and incubated with anti-POD peroxidase for 30 minutes. After washing with stop buffer, the samples were stained with diaminobenzidine (DAB) for 5 minutes and washed 3 times with PBS. For background staining, the samples were stained with Mayer's hematoxylin for 1 minute. After washing with distilled water, they were cover slipped with occlusion medium and examined by light microscopy. After staining, nuclei in 100 cells counted in 5 fields in each group were considered as TUNEL-positive cells, and the ratio was expressed as a percentage.

Immunocytochemical Staining

After the fixation of all of the groups of cells with 4% paraformaldehyde, 3% hydrogen peroxidase for 10

minutes was added and washed with PBS. Permeabilization was performed with 0.1% Triton X-100 for 10 minutes on ice. The blocking solution was added for 1-hour, then primary antibodies caspase-3 (BT-AP01199 Bioassay Technology Laboratory, China), caspase-8 (BS-0052R Bioss antibodies, USA), caspase-9 (BS-0049R Bioss antibodies, USA), RIPK1 (5805R Bioss, USA), RIPK3 (SC-374639 Santa Cruz Biotechnology, USA), MLKL (SC-293201 Santa Cruz Biotechnology, USA), occludin (SC-133256 Santa Cruz Biotechnology, USA), claudin (AB-203563 Abcam, UK), ICAM (SC-8439 Santa Cruz Biotechnology, USA), and MadCAM-1 (365934 Santa Cruz Biotechnology, USA) were added and incubated overnight at 4 °C. After washing with PBS, biotinylated secondary antibody and horseradish peroxidase-streptavidin were added to each of them for 30 minutes. The slides were stained with DAB for 5 minutes and washed with PBS and then with distilled water. After Mayer's hematoxylin staining for 1 minute, the slides were mounted with a mounting medium. The immunoreactivity density was scored as negative (-), mild (+), moderate (++), or severe (+++), and the H scores were calculated according to the immunohistochemical staining results. The formula ∑Pi (I+1) was used for the H-score (I: staining intensity, Pi represents the percentage of stained cells for each intensity).

Statistical Analysis

In the analysis of the data obtained from all the parameters studied, the differences between the groups were examined using Graphpad Prism 9 and the non-parametric one-way ANOVA test. P values less than 0.05 were considered statistically significant.

Results

MTT Results

At the 48-hour of incubation mark, the cell viability rate of 106 µL/cm^2 dose (diluted 2:1) of sucralfate was 1.8 times higher than the control group. This showed that at 48 hours, sucralfate application had led to the loss of approximately 20% of the cells. However, cell loss was slightly higher at other doses and dilutions (Figure 1). Therefore, the 106 µL/cm^2 dosage was chosen in this study.

Model of NEC Established in IEC-6 Cells

The confirmation of the NEC model, TNF-α and IL-8 levels after 24 and 48 hours of application of LPS were evaluated. The highest level of TNF-α was obtained at 48 hours of LPS incubation (Figure 2).

TUNEL Staining

While TUNEL positive cells were 13.8±2.77% in the control group, they were 65.6±8.26% in the NEC Group. TUNEL positive cells were found to be lower in the treatment and prophylaxis groups with respect to the NEC group ($p<0.05$) (Figure 3).

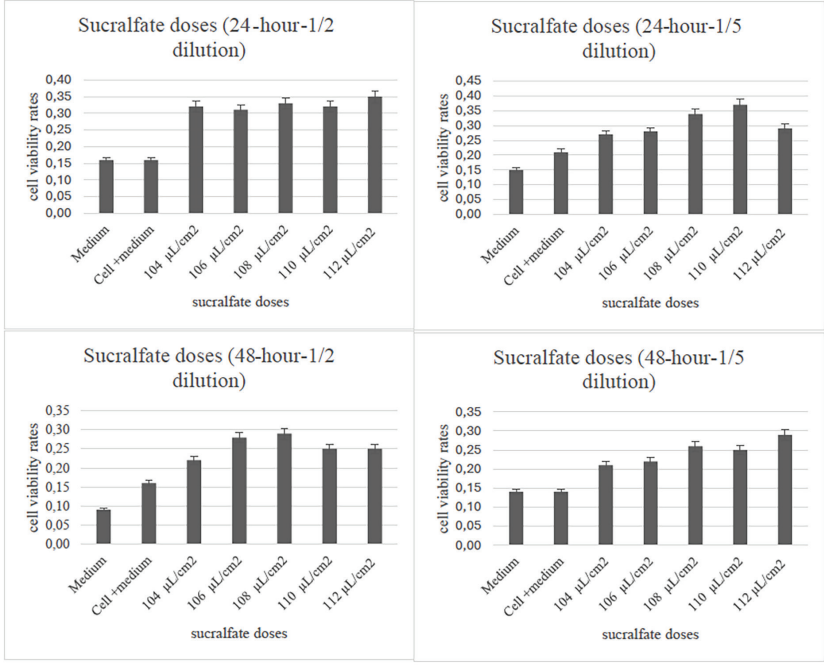

Figure 1. Effect of different dilutions and doses of sucralfate on cell viability rates (MTT test). At the 48th hour of incubation, the cell viability rate of 106 µL/cm^2 dose (diluted 2:1) of sucralfate was 1.8 times higher than the control group

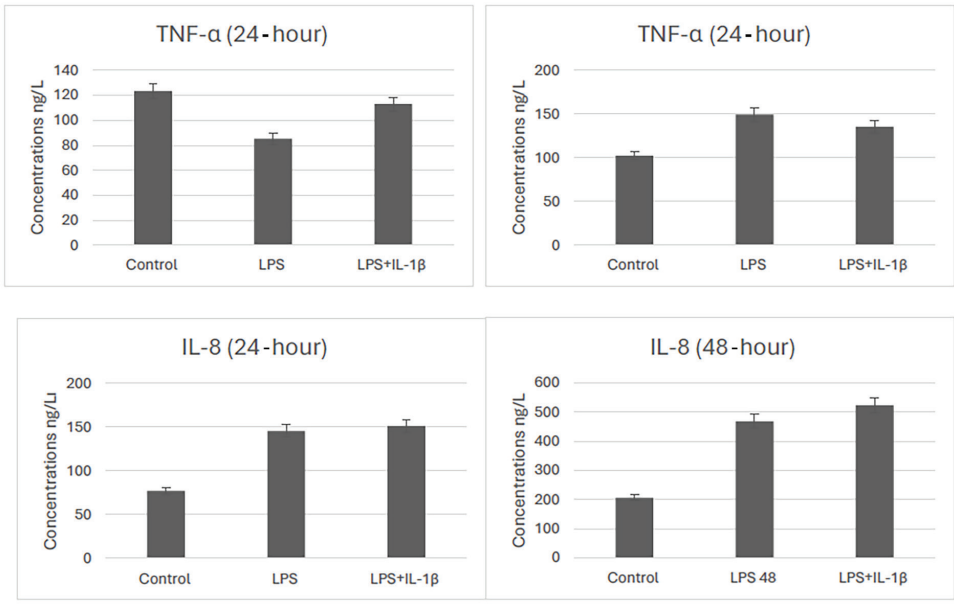

Figure 2. TNF-α and IL-8 levels triggered by lipopolysaccharide (LPS) and LPS +IL-1β (Interleukin 1β) added to enterocyte cell culture for 24 and 48 hours (TNF-α and IL-8 levels, which reached approximately 2-fold compared to the control group, were obtained only at the 48th hour of LPS application)

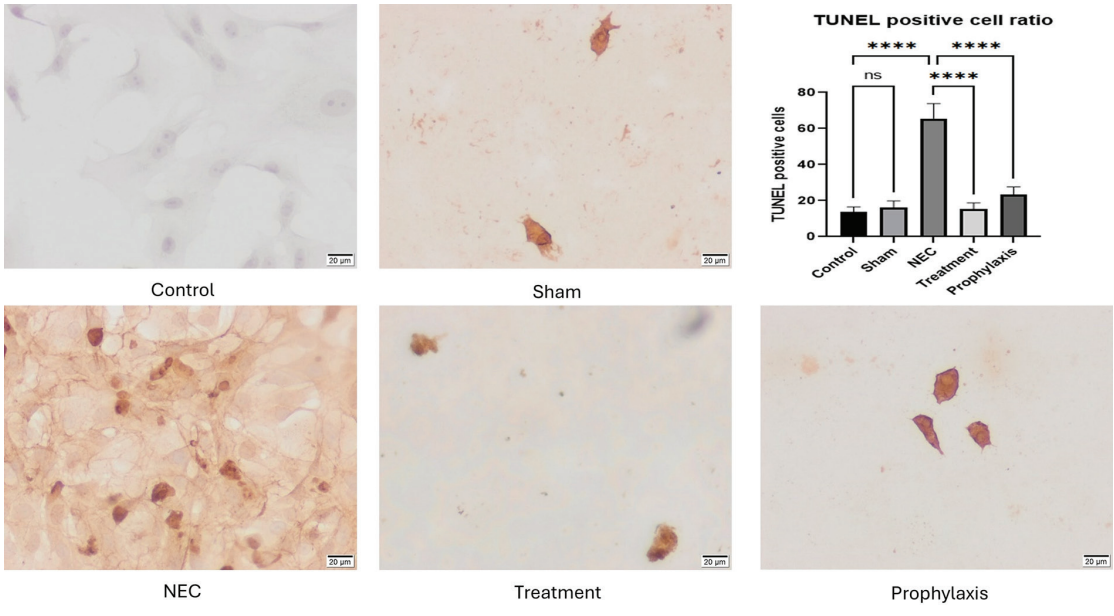

Figure 3. TUNEL (+) cell rates of the experimental groups are seen in the graph. TUNEL (+) cell rate was significantly lower in the treatment and prophylaxis groups than in the NEC group (NEC: Necrotizing enterocolitis, TUNEL: Terminal deoxynucleotidyl transferase dUTP end-labeling, ****p<0.0001, ns: non-significant). In immunohistochemical staining, TUNEL (+) cells were very dense in the NEC group compared to the control group, while the density was less in the treatment and prophylaxis groups (x20 μ)

Immunohistochemistry Staining

Apoptosis

Although the distribution of caspase 3 was lower in the treatment group than in the NEC group, it was not statistically significant (p>0.05). Caspase-3 immunoreactivity in the prophylaxis group was similar to the NEC Group (p>0.05). While the intensity of caspase 8 was higher in the NEC group than in the control group (p<0.05), in the treatment and prophylaxis group, this immunoreactivity was lower than in the NEC group but statistically not significant (p>0.05). The intensity of caspase 9 was significantly different in the

NEC group than in the control group (p<0.05), although it was slightly lower but not significantly so in prophylaxis or treatment groups (Figure 4).

Necroptosis

RIPK1 immunoreactivity was higher in the NEC group in comparison to the control group (p<0.05). The intensity of RIPK1 in the both the treatment and prophylaxis groups was similar to the NEC group. However, the immunoreactivity of RIPK3 in the both treatment and prophylaxis groups was significantly less than that in the NEC group (p<0.05) (Figure 5).

MLKL immunoreactivity was increased in the NEC group compared to the control group (p<0.05). Sucralfate application decreased MLKL intensity in both the treatment and prophylaxis groups (p<0.05) (Figure 5).

Cell Adhesion Molecules

LPS application did not change ICAM-1 distribution in the NEC group. When both the treatment and prophylaxis groups were compared with the NEC group, there was no significant difference in ICAM-1 immunoreactivity. MadCAM-1 intensity was significantly higher in the NEC group than in the (p<0.05). Sucralfate administration decreased MadCAM-1 immunoreactivity in both the treatment and prophylaxis groups (p<0.05). Occludin intensity was higher in the NEC and treatment groups in comparison to the control group (p<0.05). The immunoreactivity of occludin was slightly less in the prophylaxis group (p<0.05) (Figure 6).

While claudin intensity was similar in the control and the NEC group, however, it was decreased significantly in the treatment group (p<0.05) (Figure 6).

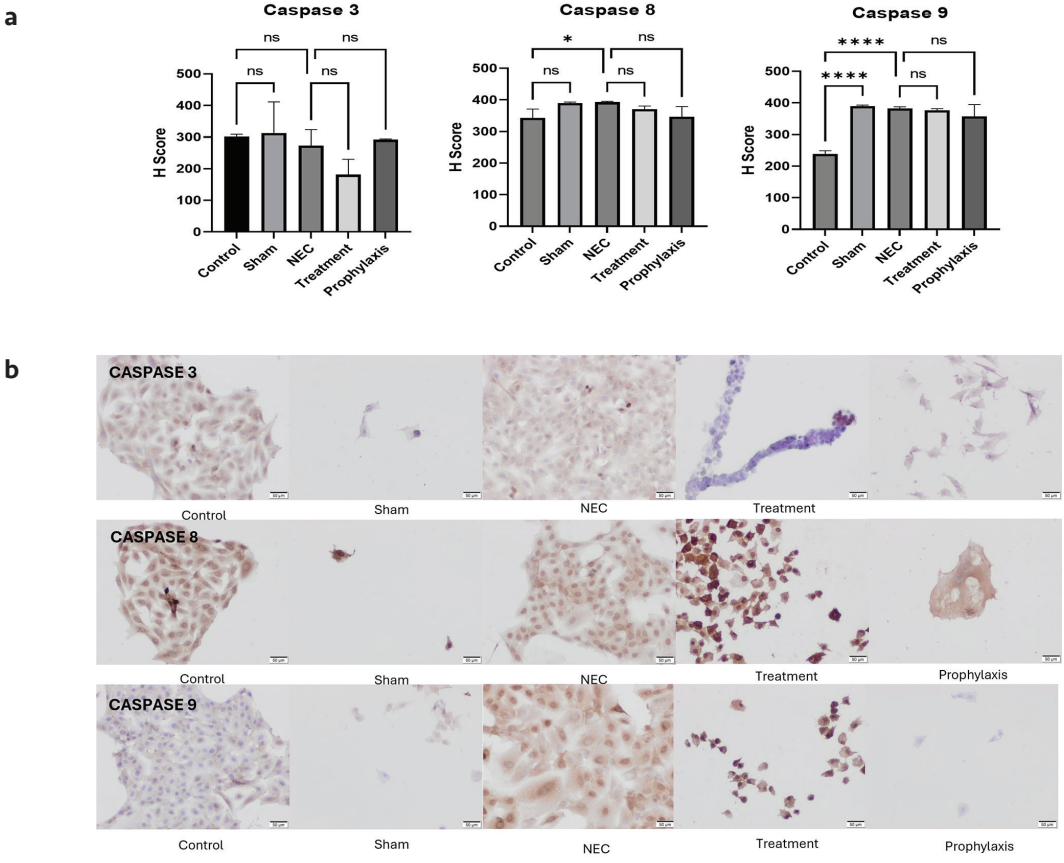

Figure 4. a) The H scores of caspase 3, 8 and 9 are seen in the graph. Especially when the treatment and prophylaxis groups were compared with the NEC group, although the H score of Caspase 3 was lower in the treatment group, no significant difference was found between the groups. When the NEC group was compared with the control group, the H scores of caspase 8 and 9 were significantly higher in the NEC group (*p<0.05, ****p<0.0001, ns: non-significant). b) Immunohistochemical staining of caspase 3,8,9 can be seen at the bottom. Here, especially in the NEC group, caspase 8 and 9 immunoreactivities are seen to be stained more intensely. Sham groups showed lower caspase 3, 8 and 9 immunoreactivities and cell density compared to control groups (x50 µm)

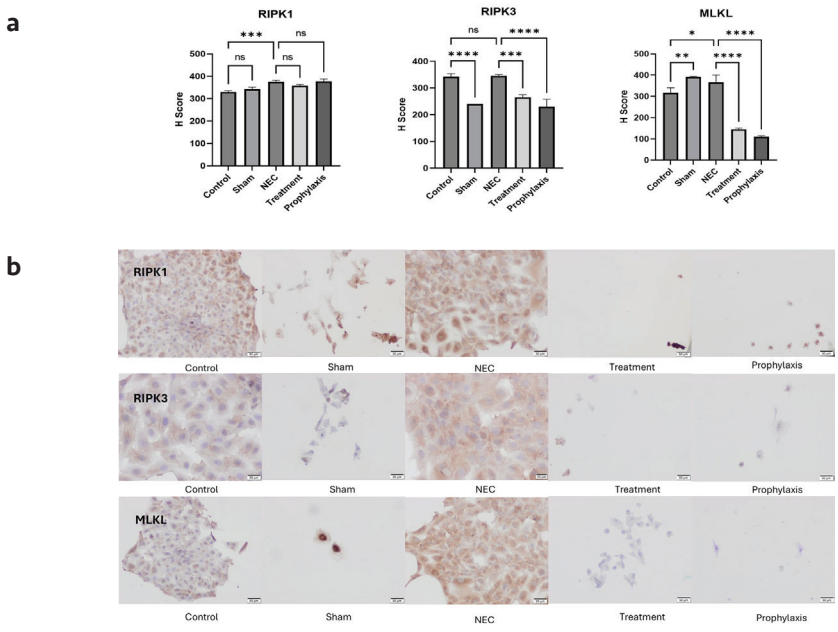

Figure 5. a) Evaluation of the necroptotic pathway. The H score results of RIPK1, RIPK3 and MLKL are shown in the graph above. When the treatment and prophylaxis groups were compared with the NEC groups, the H scores of RIPK3 and MLKL were found to be significantly lower. When the NEC groups were compared with the control groups, the H scores of RIPK1 and MLKL were significantly higher. (*p <0.05, **p<0.01, ns: non-significant). b) The immunohistochemical staining results are shown below (x50 µm). While RIPK1 and MLKL immunoactivity density was higher in the NEC group, RIPK3 and MLKL density was found to be less intense in the treatment and prophylaxis groups. Sham groups showed lower RPIK1, RIPK3 and MLKL immunoreactivities and cell density compared to control groups (x50 µm)

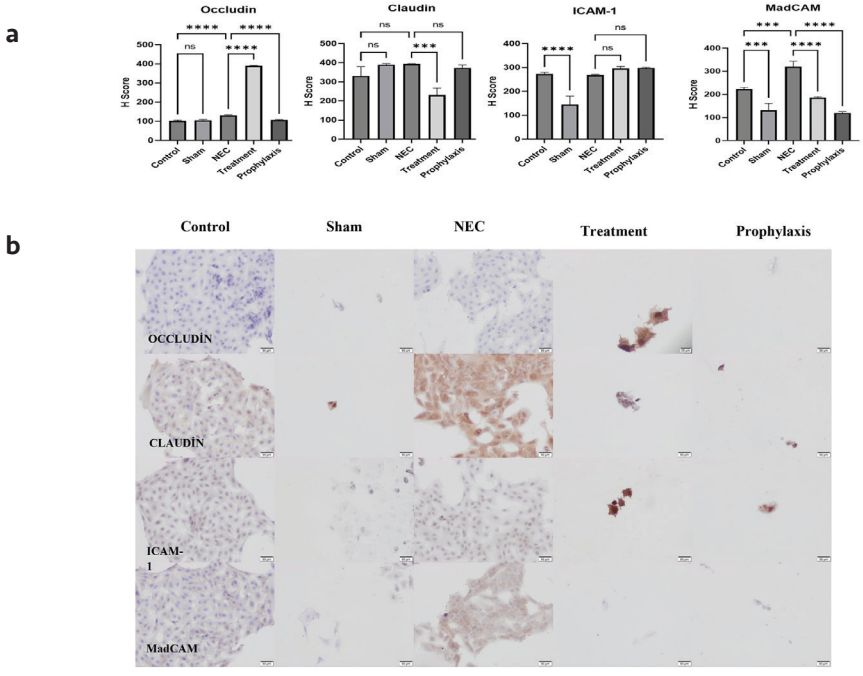

Figure 6. a) The graph (top) shows the H scores of cell adhesion molecules (Occludin, claudin, MadCAM-1, ICAM-1) in the experimental groups. H scores of the occludin were significantly higher in the treatment and prophylaxis groups than in the NEC group, while they were lower in the prophylaxis group. When the NEC group was compared with the control group, H scores of the occludin were higher in the NEC group. Claudin H scores were significantly lower only in the treatment group. ICAM-1 H scores did not differ significantly between the treatment and prophylaxis groups (***p<0.001, ****p<0.0001, ns: non-significant). b) In the lower part, immunohistochemical staining of cell adhesion molecules can be seen (x50µm). The treatment group showed a more intense occludin immunoreactivity compared to the NEC group, while claudin density was lower. The density of MadCAM was also lower in both the treatment and prophylaxis groups compared to the NEC group

Discussion

NEC predominantly affects premature infants and it is thought to result from a disruption of the intestinal mucosal barrier and bacterial colonization, leading to high morbidity and mortality (1,7). NEC appears to be the final common pathway of various pathologies causing inflammatory bowel disease in newborns (8-10).

Although many agents have been tested in NEC experimental models, none have entered clinical practice. Some studies suggest breast milk reduces NEC incidence, and agents that support the intestinal barrier may be effective treatments. Sucralfate, which protects the intestinal mucosal barrier, may have therapeutic or prophylactic effects in NEC.

Enterocytes can express proinflammatory cytokines such as IL-6, IL-8, IL-1β, TNF-α, δ-INF, and GM-CSF under different conditions (6,7). IL-1β and LPS (endotoxins) disrupt the intestinal barrier, reduce intestinal villus crypt formation, and increase permeability (7,11,12). We evaluated inflammatory response (TNF-α and IL-8 levels) by applying LPS and LPS+IL-1β to intestinal epithelial cell cultures for 24 and 48 hours. Higher levels of TNF-α and IL-8 were observed with 48-hour LPS administration compared to LPS+IL-1β, leading us to perform the NEC model with 48-hour LPS incubation alone.

Previous studies have demonstrated that oral sucralfate reduced the severity of intestinal damage in a neonatal rat NEC model and suppressed apoptosis in a rat intestinal ischemia-reperfusion model (4,5). However, these studies did not clarify how much of the sucralfate dose reached the damaged intestine, and effects beyond apoptosis were not evaluated. In this study, both apoptosis and necroptosis were observed in enterocytes in an *in vitro* NEC model.

In our study, TUNEL (+) cells were significantly reduced by sucralfate treatment and prophylaxis, indicating effectiveness in both groups. However, TUNEL positivity may also reflect necroptotic cells (13). Caspase 8 and 9 concentrations were significantly higher in the NEC group, suggesting apoptosis had begun but not been completed, as caspase 3 levels remained unchanged. Sucralfate did not significantly affect caspase 3, 8, or 9 levels.

RIPK1, a key molecule in necroptosis, was significantly increased in the NEC group, most likely due to TNF-α activation of cell death receptors. Sucralfate reduced RIPK1 levels in the treatment group, suggesting partial effectiveness, possibly by forming a protective layer. Despite this, persistent inflammation could lead to RIPK3 activation and necroptosis. Activated RIPK3 and MLKL form the necrosome complex. Phosphorylation occurs and the cell goes into necroptosis (14,15). The increase in MLKL in the NEC group indicated necroptosis, while sucralfate reduced both RIPK3 and MLKL levels, supporting its cytoprotective effect.

Tight junction proteins like occludin and claudin play crucial roles in maintaining intestinal mucosal barrier integrity (16,17). Occludin expression, reduced in NEC, was significantly increased by sucralfate, suggesting its benefit in preserving cell integrity. In the literature, it has been shown that occludin expression is reduced in NEC (18-20). Claudin regulates fluid ion diffusion between cells and, together with occludin, closes the gap between adjacent cells. Claudins are also associated with the actin cytoskeleton (20,21). Claudin density was higher in the NEC group but not statistically significant. Sucralfate's cytoprotective effect was observed in the treatment group but not in the prophylaxis group. Ares et al. (22) it was suggested that claudin-2 traffic to the cytoskeleton increased due to increased cell permeability in NEC, and therefore claudin-2 expression increased in NEC. The increase in claudin in the NEC group may have occurred for a similar reason.

ICAM-1, an important regulator in pathological conditions (23,24), showed no significant difference between the NEC and control groups, nor between the treatment and prophylaxis groups, suggesting LPS stimulation does not affect ICAM-1 in IEC-6 cells. Sumagin et al. (25) reported that TNF and LPS induced ICAM-1 expression in endothelial cells but not in IEC-6 cells.

MadCAM-1, an adhesion molecule induced in inflammatory diseases (23), was significantly higher in the NEC group. Sucralfate significantly reduced MadCAM-1 expression, suggesting it helps maintain cell integrity in the NEC model.

Study Limitations

Our study had certain limitations. Incubation with sucralfate for 48 hours reduced cell numbers in some experimental groups, possibly due to the high viscosity of the sucralfate coating the cells and reducing contact with the culture medium. Additionally, a difference was observed in the H scores of some cell adhesion molecules in the sham group compared to the control group. Although we could not fully explain this situation, we thought it might be related to the viscosity of sucralfate.

Conclusion

In conclusion, our study showed changes in apoptosis, necroptosis, and cell adhesion molecules in an *in vitro* NEC model. Sucralfate appears to protect intestinal epithelial

cells by regulating apoptotic-necroptotic activity and cell adhesion molecules. However, its prophylactic effect is less pronounced than its therapeutic effect. Further studies in other NEC models are needed to confirm sucralfate's effects. The involvement of apoptosis and necroptosis in NEC pathogenesis suggests potential future treatments using inhibitors of these pathways.

Ethics

Ethics Committee Approval: This study's design was approved by the Health Sciences Ethics Committee of Manisa Celal Bayar University Faculty of Medicine (date: 02.06.2021, approval no.: 20.478.486/842).

Acknowledgments: I would like to thank Damla Akoğulları and Hilal Kabadayı Ensarioğlu for their help during laboratory studies.

Footnotes

Authorship Contributions

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

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

Financial Disclosure: It was funded by Manisa Celal Bayar University Scientific Research Projects Office (2021-073).

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J Pediatr Res 2024;11(4):198-206
DOI: 10.4274/jpr.galenos.2024.42800

Original Article

Effects of the STING R232/H232 Variant on the Prognosis of Inflammatory Bowel Disease

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ABSTRACT

Aim: Inflammatory bowel disease (IBD) refers to a group of diseases which cause chronic and recurrent inflammation in different parts of the digestive tract, such as Crohn's disease (CD) or ulcerative colitis (UC). CD can affect both the large and small intestines, while UC usually affects only the large intestine. Recent studies in immunogenetics have revealed that the innate immune system is crucial in triggering gut inflammation, and rare variants in genes which function in this system are important risk factors for this disease. Stimulator of interferon genes (STING) is a nucleotide-binding endoplasmic reticulum protein involved in the innate immune response. This study aimed to analyse the R232/H232 variant in the *STING1* gene in pediatric patients diagnosed with IBD and to investigate whether this variant is associated with the prognosis of IBD.

Materials and Methods: Thirty-five pediatric patients admitted with a prediagnosis of IBD were included in this study. The R232/H232 variant was determined by end-point genotyping analysis after real-time reverse transcription-polymerase chain reaction (qRT-PCR) reactions using affinity probes. qRT-PCR analyses were performed to determine the mRNA expression levels of STING and interferon-induced genes in tissue samples. The western blotting method determined STING expression at the protein level.

Results: It was determined that 31.43% of the patients had heterozygous (R232/H232), and 68.57% had homozygous (H232/H232) genotypes. A significant difference was found between the genotype distribution and treatment stage. It was determined that 87.50% of the patients who started second-stage treatment had homozygous genotypes. It was also found that homozygous patients had longer durations of attacks than heterozygous patients.

Conclusion: R232/H232, the most common variant in the *STING1* gene, affects treatment response and attack duration in patients with IBD. Therefore, we suggest that variants in the *STING1* gene may be used to develop genetic-based personalized treatment strategies for IBD patients in the future.

Keywords: STING, R232/H232, inflammatory bowel disease, Crohn's disease, ulcerative colitis

Introduction

Inflammatory bowel disease (IBD) is a chronic gastrointestinal condition encompassing Crohn's disease (CD), ulcerative colitis (UC), and unclassified colitis (IBDU). The development of IBD is influenced by a combination of factors, including intestinal microbiota, environmental stimuli, immune responses, and genetic predisposition. CD is characterized by transmural inflammation and can occur anywhere in the gastrointestinal tract, from the oral cavity to the anus (1). UC is defined as a chronic inflammatory condition which causes continuous mucosal inflammation, affecting the rectum and various regions of the colon (2).

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Received: 13.09.2024 **Accepted:** 28.10.2024

IBDU refers to IBD cases confined to the colon which do not fit the specific characteristics of CD or UC (2). IBD can exhibit significant variability in terms of disease severity and prognosis; some patients may achieve remission, while others may experience relapse and progress to more complex phenotypes (3,4).

Currently, there is no definitive cure for IBD; therefore, the primary goal of treatment is to achieve long-term clinical remission without disease progression. Therapeutic strategies for CD aim to prevent complications such as structuring disease, penetrating disease, or the need for surgery. In UC, the focus is on reducing acute and chronic inflammation in order to prevent complications, avoid progression to surgery, and achieve remission (5).

The standard treatment approach involves a step-up therapy, where treatments are escalated based on disease severity. Initially, patients are treated with 5-aminosalicylic acids, such as mesalamine or sulfasalazine, or corticosteroids, including budesonide, prednisone, or dexamethasone. However, corticosteroids cannot be used continuously; therefore, in cases of disease worsening, the treatment plan may include immunosuppressive agents such as azathioprine (AZA) and mercaptopurine (MP), cyclosporine, methotrexate (MTX), or biological agents in order to maintain remission. However, all immunosuppressive medications have limited efficacy in inducing remission and are beneficial to fewer than half of those patients suffering from steroid dependency or resistance (6).

Future treatment strategies should incorporate therapeutic options tailored to the genetic profiles of the patients. Genome-wide association studies (GWAS) have identified over 163 single nucleotide variants (SNVs) associated with IBD (7,8). An example is a variant in the *neutrophil cytosolic factor 4* (*NCF4*) gene, which has been identified as a risk gene for CD. Although this SNV is not located in the coding region, it causes functional impairment in granulocytes (8). In CD patients with this variant, targeted treatments which stimulate granulocytes, such as sargramostim, have been shown to improve remission (9). Determining the correlations between genetic makeup, patient characteristics, and treatment responses will increase the likelihood of implementing personalized treatment strategies. Despite the availability of numerous drugs for IBD treatment and the introduction of new medications, none have proven universally effective for all IBD patients (10).

Stimulator of interferon genes (STING) is an endoplasmic reticulum protein involved in innate immune signalling. The STING protein, encoded by the *STING1* gene located on chromosome 5 (gene ID: 340061), is also known by names such as MITA, STING, hSTING, and TMEM173. It is a key inducer of type I interferons which are produced in response to cytosolic DNA or bacterial cyclic dinucleotides and play a canonical role in antiviral and antibacterial immunity (11). STING functions, such as being a fundamental mediator of innate immune responses to microbial and host-derived DNA, is crucial in sensing and regulating responses to infection, cellular stress, and tissue damage (12). Studies have also highlighted STING's essential role in gastrointestinal homeostasis and its significant overlap with IBD pathophysiology (13). Additionally, fundamental findings related to the structural and molecular biology of the cGAS-STING pathway have enabled the development of selective small molecular inhibitors with potential targeting capabilities for a range of inflammatory diseases in humans (14). A variant in the *STING1* gene, causing either arginine (R) or histidine (H) at position 232, has been identified (rs1131769). Cytosine is the most frequently observed nucleotide at this position (4425), making the R232 allele the wild-type variant (15). This study aimed to analyse the R232/H232 variant in pediatric patients diagnosed with IBD and to investigate whether this variant is associated with the prognosis of IBD.

Materials and Methods

Patient Population

This study, approved by our centre's Ethics Committee of Ege University (approval number: 21-12.1T/26, date: 21.04.2022), included a cohort of 35 pediatric patients, aged 0-18 years, diagnosed with IBD at the department of pediatric gastroenterology. During colonoscopy, biopsy specimens and 2 mL of blood samples were collected from each patient. The patients, diagnosed based on clinical and histopathological criteria, initially received steroid therapy aimed at inducing remission, followed by a maintenance regimen. Steroids were administered for 4-6 weeks, after which treatment transitioned to a maintenance phase with a gradual reduction in steroid dosage. Those patients demonstrating a positive response to the therapy were subsequently monitored with follow-up visits at 2, 4, and 8 weeks, and then at 3-month intervals.

Isolation Procedures

For DNA extraction from the blood samples, the Nucleic Acid Isolation Kit I (Roche Diagnostics, Mannheim, Germany) was employed. From 400 µL of blood placed into the MagNA Pure Compact (Roche Diagnostics, Mannheim, Germany) device, a total volume of 100 µL of DNA samples was obtained, with an average concentration of 80 ng/µL.

RNA isolation from the tissue samples was performed using tripure solution (Roche Diagnostics, Mannheim, Germany). A 5 mg tissue sample was homogenized in tripure solution. After the addition of chloroform, the mixture was centrifuged at 12,000 xg for 45 minutes to achieve phase separation. The upper clear phase was transferred to a separate Eppendorf tube for RNA precipitation using ethanol. The purity and concentration of the isolated nucleic acids were assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA).

For protein isolation from the tissue samples, the complete™ Lysis-M kit (Roche Diagnostics, Mannheim, Germany) was used. Protein concentrations were determined spectrophotometrically using Bradford reagent and bovine serum albumin (BSA) standards (Fermentas, Massachusetts, USA).

Genotyping Analysis

The R232/H232 variant was determined using end-point genotyping analysis following real-time polymerase chain reaction (PCR) with affinity probes on the LightCycler 480 system (Roche Diagnostics, Mannheim, Germany). Primers and probes were obtained from Integrated DNA Technologies (IDT, Iowa, USA). The primer and probe sequences used in this analysis were as follows:

- **R232H Forward Primer:** 5'CGTTCTCCAGAAGCTCATAG3'
- **R232H Reverse Primer:** 5'CCCAACATTCGCTTCCT3'
- **Wild-Type Genotype Probe:** 56-FAM/CA+GC... G+T+CA/ 3IABkFQ
- **Mutant Genotype Probe:** 5HEX/AGC +A+C+G G+T+C A/3IABkQ

Genotype Validation via Sanger Sequencing

In order to validate the results obtained from the genotyping analyses, 1-2 samples from each genotype were selected for confirmation using the Sanger sequencing method. The specific region of the *STING1* gene containing the R232/H232 variant was amplified using specific primers (forward primer: 5' TCATCAGTGCTTGGCTAGG 3'; reverse primer: 5' CTTCCCTGCCTCAGAGCTPCR 3'). The amplified products were visualized through agarose gel electrophoresis. The PCR products were purified using the NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany) and subjected to Big Dye PCR for Sanger sequencing. After a second purification of the PCR products, sequencing was performed.

Gene Expression Analyses via Real-time Reverse Transcription-PCR (qRT-PCR)

All RNA samples were diluted to a final concentration of 70 ng and subsequently reverse transcribed into cDNA using the iScript™ cDNA Synthesis Kit (Biorad, California, USA). To assess gene expression levels, qRT-PCR was performed on the LightCycler 480 system using iTaq Universal SYBR Green Supermix (Biorad, California, USA) and gene-specific primers. The primer sequences used for this analysis are provided in Table I.

Western Blot Analysis

In order to determine the expression of STING at the protein level, western blot analysis was performed following protein isolation from the tissue samples. Protein samples were separated on an SDS-PAGE gel at a concentration of 10 µg/mL. After the transfer and blocking steps, the membrane was incubated overnight with a primary STING antibody (#13647S, Cell Signaling, Massachusetts, USA) diluted 1:1000 in a 5% BSA solution. A horseradish peroxidase-conjugated anti-rabbit secondary antibody (#7074, Cell Signaling, Massachusetts, USA) was used at a 1:1000 dilution. Following several washes, the membrane was

Table I. Forward and reverse primer sequences used in gene expression analyses

Primer	Base sequence 5'-3'
STING-F	GCAGTGTGTGAAAAAGGGAAT
STING-R	CACCCCGTAGCAGGTTGTT
IFNβ-F	CAGCATCTGCTGGTTGAAGA
IFNβ-R	CATTACCTGAAGGCCAAGGA
IFIT2-F	GCGTGAAGAAGGTGAAGAGG
IFIT2-R	GCAGGTAGGCATTGTTTGGT
IFI44-F	GATGTGAGCCTGTGAGGTCC
IFI44-B	CTTTACAGGGTCCAGCTCCC
IL6-F	AGACAGCCACTCACCTCTTCAG
IL6-R	TTCTGCCAGTGCCTCTTTGCTG
ISG15-R	CAGCCATGGGCTGGGAC
ISG15-F	GCCGATCTTCTGGGTGATCT

STING: Stimulator of interferon genes, *IFNB*: Interferon beta, *IFIT2*: Interferon induced protein with tetratricopeptide repeats 2, *IFI44*: Interferon-induced protein 44, *IL6*: Interleukin 6, *ISG15*: Interferon-Stimulated gene 15

incubated with an enhanced chemiluminescence solution (Biorad, California, USA), and the signal was detected using a C-Digit blot scanner (Li-Cor Biosciences, Nebraska, USA).

This procedure enables the quantification and visualization of STING protein expression in tissue samples.

Statistical Analysis

The patients were stratified based on their genotypes, and subsequent comparisons were made across various prognostic markers including gene/protein expression levels, treatment response, frequency and duration of relapses, and time to remission. Statistical analyses were conducted using SPSS software (version 26). Fisher's exact test was employed to compare allele frequencies between the groups. The distribution of gene expression levels was assessed using the Kolmogorov-Smirnov and Shapiro-Wilk tests to evaluate normality. For non-normally distributed data, differences between groups were analysed using the Mann-Whitney U test. A p-value of less than 0.05 was considered statistically significant.

Results

Clinical Data

This study included a total of 35 patients, comprising 16 males (45.71%) and 19 females (54.29%). Based on their clinical findings, laboratory results, endoscopy, and colonoscopy outcomes, 30 patients (85.71%) were diagnosed with UC and 5 patients (14.29%) were diagnosed with CD. Clinical characteristics, including follow-up durations, relapse frequencies and durations, treatments, treatment responses, and times to remission, are summarized in Table II.

Genotype Findings for R232/H232

The genotype analysis revealed that 15.71% of the patients carried the C allele, whereas 84.29% carried the T allele. Consequently, among the 35 patients, 11 were identified as heterozygous (CT, R232/H232), representing 31.43% of the cohort, and 24 were homozygous (TT, H232/H232), accounting for 68.57%. These genotype classifications were corroborated through Sanger sequencing, which confirmed the results obtained using the LightCycler 480 device, as illustrated in Figure 1.

Upon stratifying the IBD patients by disease type and genotype, it was found that all individuals with CD exhibited the homozygous genotype (H232/H232, 100%). In contrast, among those patients with UC, 11 individuals were heterozygous (R232/H232, 36.67%), while 19 were homozygous (H232/H232, 63.33%) (Table III).

Table II. Clinical data of the included patients

Gender	Female	19 (54.29%)
	Male	16 (45.71%)
Etiology	UC	30 (85.71%)
	CD	5 (14.29%)
Follow-up duration	<1 year	4 (11.43%)
	1-3 years	21 (60.00%)
	4-5 years	7 (20.00%)
	>5 years	3 (8.57%)
Treatment response	First stage	19 (54.29%)
	Second stage	16 (45.71%)
Attack duration	1-3 day	17 (48.57%)
	≥4 day	18 (51.43%)
Number of attacks	1	13 (37.14%)
	2	5 (14.29%)
	3	4 (11.43%)
	≥4	13 (37.14%)
Time to remission	1 day	7 (20.00%)
	2 days	3 (8.57%)
	3 days	15 (42.86%)
	≥4 days	10 (28.57%)

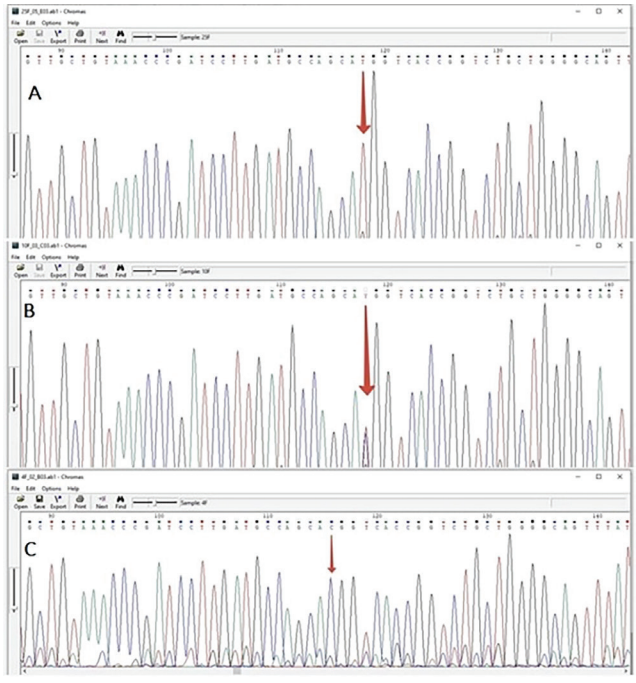

Figure 1. Genotype validation by sanger sequencing A: Wild-type (R232/R232), B: Heterozygous (R232/H232), C: Homozygous (H232/H232)

Statistical analysis revealed no significant differences in the distribution of heterozygous and homozygous genotypes across the various disease subgroups.

Comparison of R232/H232 Genotype with Prognostic Markers

After stratifying the patients according to their genotypes, prognostic markers such as treatment response, the number of relapses within one year, relapse duration, and time to remission following treatment were evaluated and subjected to statistical analysis.

In our study, when comparing the patients undergoing first and second-phase treatments based on their genotypes, it was observed that a significant proportion of those advancing to the second-phase treatment (87.5%) had the homozygous genotype. This analysis, which included all patients, found a significant difference in treatment response based on the R232/H232 genotype ($p<0.027$) (Table IV).

Comparison of Relapse Duration and Frequency Based on R232/H232 Genotype

A comparison of relapse durations across R232/H232 genotypes revealed a statistically significant difference ($p<0.009$) (Table V). Specifically, among those patients experiencing relapses lasting four days or more, 11.11% had the heterozygous genotype, whereas 88.89% had the homozygous genotype.

When the analysis was restricted to patients with UC, a similar trend was observed: 84.61% of those with relapses lasting four days or more carried the homozygous genotype. However, the p-value was 0.058, which is on the threshold of statistical significance (Table VI).

Further analysis of relapse frequency throughout the year did not reveal significant differences based on the R232/H232 genotype, indicating no meaningful variation in relapse counts between heterozygous and homozygous cases. Additionally, no significant differences were found between the time to remission and the distribution of R232/H232 genotypes.

Effects of R232/H232 Genotype on cGAS/STING Pathway Components

In our study, the expression of STING at both mRNA and protein levels was analysed in the biopsy tissues from the patients with IBD. Comparative analysis of STING expression between heterozygous and homozygous genotypes revealed no significant differences (Figure 2, Table VII). Our findings indicate that STING protein is expressed in a significant proportion of IBD cases. However, very low levels of STING protein expression were observed in a small subset of patients (samples 7, 8, and 29) (Figure 2).

Additionally, expression levels of interferon-stimulated genes (ISGs) including IFNB, IFIT2, IFI44, IL6, and ISG15 were assessed. No significant differences in the expression of these genes were found between heterozygous and homozygous

Table III. Distribution of genotypes and disease subgroups among IBD cases

Disease type	Heterozygous R232/H232 (n/%)	Homozygous H232/H232 (n/%)	Total (n/%)	p*
Crohn disease	0 (0.0)	5 (100)	5 (100)	<0.157
Ulcerative colitis	11 (36.67)	19 (63.33)	30 (100)	

*Fisher's exact test, IBD: Inflammatory bowel disease

Table IV. Comparison of treatment responses based on genotype

Treatment	Heterozygous R232/H232 (n/%)	Homozygous H232/H232 (n/%)	Total (n/%)	p*
First stage	9 (47.36)	10 (52.63)	19 (100)	**<0.027**
Second stage	2 (12.50)	14 (87.50)	16 (100)	

*Fisher's exact test

Table V. Relapse durations among all patients by genotype

Duration of attack (days)	Heterozygous R232/H232 (n/%)	Homozygous H232/H232 (n/%)	Total (n/%)
1	0 (0.0)	2 (100.0)	2 (100)
2	4 (66.67)	2 (33.33)	6 (100)
3	5 (55.56)	4 (44.44)	9 (100)
>4	2 (11.11)	16 (88.89)	18 (100)

p<0.009 (Fisher's exact test)

Table VI. Relapse durations among ulcerative colitis patients by genotype

Duration of attack (days)	Heterozygous R232/H232 (n/%)	Homozygous H232/H232 (n/%)	Total (n/%)
1	0 (0.0)	2 (100)	2 (100)
2	4 (66.67)	2 (33.33)	6 (100)
3	5 (55.56)	4 (44.44)	9 (100)
>4	2 (15.38)	11 (84.61)	13 (100)

p<0.058 (Fisher's exact test)

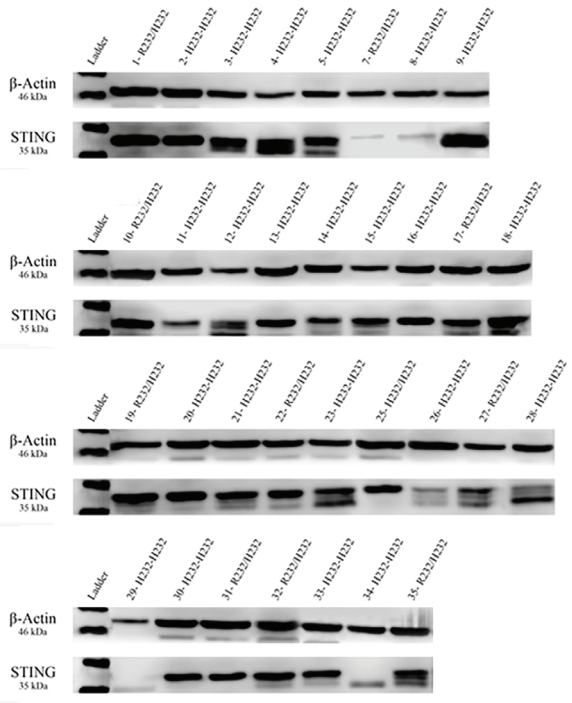

Figure 2. Western blot gel images for B-Actin and STING protein in biopsy samples from IBD patients. The first line indicates the ladder. Genotypes are indicated on the top of the gels and each line represents a patient
STING: Stimulator of interferon genes, IBD: Inflammatory bowel disease

Table VII. Comparison of gene expression levels between heterozygous and homozygous genotypes in all IBD patients

Gene	Genotype	Expression level (mean ± SD)	Minimum	Maximum	p*
STING	R232/H232	1.29±0.89	0.42	3.13	0.734
	H232/H232	1.16±0.89	0.22	4.24	
IFNB	R232/H232	3.047±4.858	0.18	13.93	0.913
	H232/H232	2.224±3.521	0.13	16.01	
IFIT2	R232/H232	1.467±1.814	0.02	5.77	0.722
	H232/H232	2.881±4.783	0.16	20.64	
IFI44	R232/H232	2.066±1.599	0.18	5.01	0.160
	H232/H232	1.267±1.567	0.20	6.17	
IL6	R232/H232	3.440±5.616	0.08	18.65	0.806
	H232/H232	3.491±6.186	0.01	24.78	
ISG15	R232/H232	1.274±0.856	0.29	2.93	0.663
	H232/H232	1.983±2.966	0.17	3.18	

*: Mann-Whitney U test, IBD: Inflammatory bowel disease, SD: Standard deviation, *STING*: Stimulator of interferon genes, *IFNB*: Interferon beta, *IFIT2*: Interferon induced protein with tetratricopeptide repeats 2, *IFI44*: Interferon-induced protein 44, *IL6*: Interleukin 6, *ISG15*: Interferon-Stimulated gene 15

genotypes (Table VII). These results suggest that variations in the R232/H232 genotype do not substantially affect the overall expression of STING or related ISGs in the patient cohort studied.

Discussion

In this study, we explored the effects of the R232/H232 single nucleotide variant in the *STING1* gene on the prognosis of IBD. It is well-established that genetic factors play a significant role in IBD, with familial clustering observed in approximately 5-10% of patients. SNVs can influence disease development by affecting the production or function of proteins, which in turn can impact cellular functions, innate immune responses, and consequently both disease activity and treatment response (16,17). Despite the identification of numerous genes involved in the development of IBD, the precise mechanisms by which the SNVs in these genes affect cellular functions or contribute to IBD pathogenesis remain unclear.

GWAS have identified over 163 SNVs associated with IBD (7,18). The incidence of these SNVs in IBD populations differs from that in the general population, and the precise mechanisms by which these variants influence cellular functions or contribute to IBD pathogenesis remain inadequately understood. Some genes, such as *NOD2*, *ATG16L1*, *IL23R*, and *IRGM*, have had their cellular effects elucidated, including their impacts on innate immune cell functions, autophagy processes, and bacterial clearance (19-21).

In IBD management, steroids are not used for maintenance therapy due to their limitations. Consequently, first-line treatments often involve immunosuppressive AZA, MP, or MTX. These agents are employed to induce and maintain remission. However, their efficacy in achieving remission induction is limited, and they benefit fewer than half of those patients suffering from steroid dependence or resistance (6). Anti-tumor necrosis factor (TNF) α therapies, employed in the second phase of treatment, have demonstrated considerable effectiveness in IBD. Nevertheless, anti-TNFα therapy is not effective in approximately 30% of IBD patients, and a significant number of patients may experience loss of response or adverse effects. This often necessitates surgical intervention (22,23).

Mutations in the *STING1* gene are associated with a severe autoinflammatory disease known as STING-associated vasculopathy with onset in infancy (SAVI), with this being a life-threatening condition (24). The human *STING1* gene exhibits substantial heterogeneity and population-level

variability (24). The H232 variant, first identified in the *STING1* gene, features a histidine residue at position 232 (25-27). This H232/H232 genotype has been reported in approximately 30% of East Asians and 10% of Europeans. Structural and functional studies of the human STING protein have predominantly used the H232 allele, which is suggested to be a minor allele which may lead to functional impairment (28). *In vitro* studies have demonstrated that cells carrying the H232 allele respond less effectively to cyclic dinucleotides (29,30). Specifically, the STING protein carrying the H232 variant binds metazoan 2'3'-cGAMP but exhibits a diminished interferon response to bacterial c-di-GMP, and shows a complete loss of response to c-di-AMP and 3'3'-cGAMP (15,31).

The most prevalent allele in the population, R232, contains an arginine amino acid at position 232 and is considered the "wild-type" *STING1* isoform, found naturally in approximately 60% of the population (15). The R232/R232 genotype is dominant in European populations. In studies involving approximately 1,000 Americans, about 45% were found to have the R232/R232 genotype, whereas only about 2% carried the H232/H232 genotype (28). Additionally, over 50% of Americans possess at least one non-R232 *STING1* allele, indicating substantial heterogeneity in the *STING1* gene across human populations (28). The R232 amino acid is located within the loop region of STING which forms the binding pocket for c-di-GMP. This allele allows the binding of various cyclic dinucleotides, including c-di-GMP, c-di-AMP, and both 2'3'- and 3'3'-cGAMP (15,31). In contrast, the H232 variant, which has been associated with functional impairment of STING, results in a reduced response to cyclic dinucleotides, particularly affecting the protein's ability to activate downstream signalling pathways (24).

In our study, we found that all five patients with CD had the homozygous genotype (H232/H232) and carried the TT allele, while among the 30 patients with UC, 11 (36.67%) had the heterozygous CT (R232/H232) genotype and 19 (63.33%) had the homozygous TT (H232/H232) genotype. Despite the R232/R232 genotype being referred to as the wild-type in the literature (24), none of the IBD cases included in our study exhibited this genotype. In order to ensure the accuracy of the genotyping method used, validation studies were conducted using Sanger sequencing. The sequence analysis confirmed that the genotypes identified using affinity probes were accurate.

Study Limitations

A significant limitation of our study was the absence of a control group, which prevented us from determining whether the higher prevalence of the H232 variant is a general population phenomenon or specific to our patient cohort. Due to budget constraints and technical limitations, a control group could not be included in our study. However, we aim to continuing to collect samples from IBD patients in other projects in order to analyse *STING1* variants in larger case-control groups so as to better understand their prevalence and impact.

When comparing patients undergoing first and second-line therapies by genotype, it was notable that a significant proportion of patients advancing to second-line therapy (87.5%) were found to have the H232/H232 genotype. Furthermore, the significant difference observed between the H232/H232 genotype and the duration of flare-ups daily suggests that this variant may influence both treatment response and prognosis in IBD. This finding highlights the potential role of the *STING1* gene variant in modulating disease progression and therapeutic outcomes.

In the intestine, as in most tissues, basal levels of type I interferon are typically low. However, in response to infection or cellular damage, there is a rapid induction of type I interferon. Interestingly, a deficiency in type I interferon signalling during mucosal inflammation has been associated with increased cytokine release by effector T-cells (32). This suggests a potentially altered adaptive immune response in IBD as a result of variable type I interferon levels. While genotypes associated with excessive STING activity have been linked to autoinflammatory disorders, no specific *STING1* variants have been identified in GWAS as being directly associated with IBD (33). Nevertheless, it has been reported that the *STING1* gene is hypomethylated in the intestinal epithelium of a pediatric IBD cohort (33). This finding may account for the observed overexpression and potential hyperactivation of STING in the epithelial cells of IBD patients. In line with this, IBD patients exhibit a signature of interferon-regulated genes, and elevated levels of interferon are associated with a lack of therapeutic response. Additionally, the interferon-stimulated gene *ISG15* is highly expressed in IBD patients with active inflammation (34). However, our study did not find significant differences in the mRNA expression levels of ISGs such as IFNB, IFIT2, IFI44, IL6, and ISG15 between the heterozygous and homozygous genotypes.

This lack of observed difference in gene expression levels might be attributed to the fact that the patients were receiving immunosuppressive therapy at the time of biopsy. Therefore, we believe that evaluating gene expression in treatment-naive newly diagnosed patients may provide a

clearer understanding of the impact of *STING1* genotypes on interferon-regulated gene expression.

Conclusion

In summary, genetic variants in genes associated with IBD may be responsible for previously unidentified disease phenotypes and could offer opportunities for the development of new therapeutic strategies. The genetic profiles of IBD patients are hoped to be able to assist clinicians in making decisions regarding personalized treatment approaches in the future. For instance, modifications to treatment protocols could include more frequent clinical monitoring for those patients carrying variants associated with poor prognosis, earlier initiation of second-line therapy in those patients with prolonged time to remission, or even pre-emptive transition to second-line therapy before disease flare-ups occur. Such changes could lead to secondary benefits, such as reduced hospital admission durations, increased school attendance and academic performance, and/or decreased malnutrition.

Further research in larger patient-control cohorts is needed in order to elucidate the effects of these findings and to identify the corresponding disease phenotypes.

Ethics

Ethics Committee Approval: This study, approved by our centre's Ethics Committee of Ege University (approval number: 21-12.1T/date: 21.04.2022).

Informed Consent: His parents or legal guardians of patients provided signed informed consent.

Acknowledgments: We thank Mr. Emre Tepedelen, Field Application Manager at GenEra, for supporting the validation studies.

Footnotes

Authorship Contributions

Surgical and Medical Practices: M.K., D.B., V.B., Concept: G.A., D.B., V.B., Design: G.A., V.B., Data Collection or Processing: G.A., T.K., V.B., Analysis or Interpretation: M.K., T.K., V.B., Literature Search: G.A., M.K., V.B., Writing: G.A., M.K., V.B.

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

Financial Disclosure: This study was conducted with the support of Ege University Scientific Research Projects Coordination (Project ID: 23835).

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J Pediatr Res 2024;11(4):207-211
DOI: 10.4274/jpr.galenos.2024.69094

Original Article

Transcatheter Ductus Arteriosus Closure with Various Devices in the Pediatric Patient Group and Long-term Outcomes: Experience from a Single Center

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ABSTRACT

Aim: The emergence of advanced duct occluder devices has made transcatheter patent ductus arteriosus (PDA) closure the preferred treatment for pediatric patients. This study compared the effectiveness, safety, and long-term outcomes of various transcatheter PDA closure devices.

Materials and Methods: This study involved 320 patients aged 0 to 18 years who underwent transcatheter PDA closure at our hospital from 2004 to 2023. We retrospectively reviewed their records in order to assess procedure success, demographic information, clinical features, angiographic parameters, and complications. Patients were categorized by closure type: Group I for coil closure, Group II for Amplatzer Duct Occluder (ADO)-I closure, and Group III for ADO-II closure.

Results: In this study of 320 patients, 203 (63.4%) were female and 117 (36.4%) male. The average age was 56.5 months (±49.6), with a median weight of 15 kg (interquartile range 10.5-23 kg). The median diameter of the PDA at its narrowest point was 2.0 mm (interquartile range 2-3 mm). Ductal anatomy distribution was as follows: Type A (176 patients, 55%), type B (49 patients, 15.3%), type C (30 patients, 9.3%), type D (5 patients, 1.56%), type E (57 patients, 17.8%), and type F (4 patients, 1.25%). Arterial access was used in 263 patients (82.1%), and venous plus arterial access in 57 patients (17.8%). Closure techniques included the ADO-II in 107 cases (33.4%), ADO-I in 12 cases (3.75%), and coils in 201 cases (62%). The early closure rate was 97.5%, with initial shunt rates of 0.6% and 0.3% at one month. Device embolization occurred in 5 patients (1.87%). By the six-month follow-up, all PDAs had closed, resulting in an overall transaction success rate of 97.5%. The average follow-up period was 105.8±55 months.

Conclusion: Percutaneous closure of PDA in children is safe and effective, with a high success rate. Key factors include the patient's age, weight, duct dimensions, and the type and size of the PDA. ADO-I devices are ideal for larger defects, while coil or ADO-II devices are preferable for smaller ones. Proper patient selection is critical for successful outcomes.

Keywords: Children, patent ductus arteriosus, percutaneous PDA closure, PDA closure devices

Introduction

The ductus arteriosus is a blood vessel connecting the aorta and pulmonary artery during fetal development. It typically closes within 12 to 24 hours after birth. If it remains open, this condition is called "patent ductus arteriosus" (PDA) (1).

PDA occurs in about 0.03% to 0.08% of full-term infants and is more common in females (2). An open PDA can lead to serious complications such as heart failure, infective endocarditis, and pulmonary hypertension (3). Transcatheter closure of PDA began in 1967 with the Ivadon device (4). In the 1990s, coils were introduced for small

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Received: 16.10.2024 **Accepted:** 06.11.2024

ducts under 2.5 mm. The Amplatzer Duct Occluder (ADO), capable of closing larger PDAs up to 13 mm, was first used in 1997 and has become a standard treatment.

Various devices have since been developed for differing ductal anatomies. It is essential to select an appropriate device based on the patient's anatomy, age, and weight (5,6).

This article shares our long-term experience and compares the effectiveness and safety of different devices for transcatheter PDA closure in pediatric patients at our clinic.

Materials and Methods

This study was conducted on patients aged 0 to 18 who had transcatheter PDA closure at our hospital from 2004 to 2023. We reviewed the medical records, excluding those with other congenital heart anomalies or incomplete data. Informed consent was obtained, and this study was approved by the Medical Research Ethics Committee of Ege University (approval no.: 24-3T/74, date: 07.03.2024).

We assessed procedural success, demographic data, clinical features, angiographic parameters, and complications, categorizing patients by closure method: Group I for coil closure, Group II for ADO-I, and Group III for ADO-II.

Indications for PDA Closure

The patients in this study were chosen based on specific criteria (7,8). Closure of the PDA was primarily carried out for those with growth retardation, left atrial and ventricular enlargement, and audible murmurs. Those patients with faint murmurs but significant blood flow on echocardiograms and those without moderate to severe pulmonary hypertension were also included. Defects were assessed using transthoracic echocardiography. Those individuals with irreversible pulmonary vascular disease or high-pressure ratios were excluded.

Statistical Analysis

Statistical analyses were performed using SPSS Statistics version 26.0 (IBM Corp., 2019). Descriptive statistics included the number of units (n), percentages (%), median (M), and minimum and maximum values for categorical variables. Continuous variables were reported as mean ± standard deviation (SD) or median (range). Normally distributed data were analyzed with Student's t-test, while non-normally distributed data used the Mann-Whitney U test and the Kruskal-Wallis test. Chi-square analysis was applied to categorical data. The significance threshold was set at $p<0.05$.

Results

Between 2004 and 2023, 320 patients underwent percutaneous PDA closure at our clinic, with 203 girls (63.4%) and 117 boys (36.6%), resulting in a girl-to-boy ratio of 1.73. The mean age was 56.5 months (±49.6), and the median weight was 15 kg [interquartile range (IQR): 10.5-23 kg]. Closure methods included the ADO-II in 107 cases (33.4%), ADO-I in 12 cases (3.75%), and a coil in 201 cases (62.8%).

The median diameter of the PDA at its narrowest point was 2.0 mm (IQR 2-3 mm), with a significant difference between groups ($p<0.01$). Those patients treated with the ADO-I device had a mean PDA diameter of 4.1 mm (SD ± 2.1 mm), while those treated with the ADO-II device had a mean diameter of 2.9 mm (SD ± 0.9 mm). Overall, the mean PDA diameter was 2.2 mm (SD ± 0.6 mm).

On average, the diameter of the PDA was 1.9 mm larger for those patients using the ADO-I device compared to the coil device, while the ADO-II device showed a 0.7 mm increase. The 1.1 mm difference between ADO-I and ADO-II was not statistically significant. Ductal anatomy distribution was as follows: Type A in 176 patients (55%), type B in 49 (15.3%), type C in 30 (9.3%), type D in 5 (1.56%), type E in 57 (17.8%), and type F in 4 (1.25%). Type A ducts predominated in Groups 1 and 3, while Type B was most common in Group 2. Arterial access was used in 263 patients (82.1%), and combined venous and arterial access in 57 (17.8%). Transvenous access was more common in Group 2, while transarterial access more common in Groups 1 and 3. Closure procedures varied significantly by PDA morphology and device type ($p<0.01$), with type A ducts most frequent in Group 1 (63.2%) and Group 3 (42.1%), and type B ducts dominant in Group 2 (50%).

The PDA was successfully closed in 312 of 320 patients, achieving a 97.5% success rate. There were 8 failures (2.5%), with residual shunts in two patients on the first day. Shunt occurrence was 0.6% initially and decreased to 0.3% after one month. No residual shunts were found in the ADO-I and ADO-II device groups. By the six-month follow-up, all patients with residual shunts had their PDAs closed, leading to an overall one-year success rate of 98.1%. Device embolization occurred in 5 patients (1.87%), four of whom had been treated with coils and one with an ADO-II device.

Embolization procedures involved one coil and the ADO-II device in the main pulmonary artery, two coils in the right pulmonary artery, and one coil in the aorta. One patient required surgery to remove the embolized device and ligate the PDA, while the other four received

transcatheter treatment and later underwent PDA closure surgery due to device availability and cost issues. One patient (0.3%) experienced cardiac tamponade and needed urgent intervention. Two cases (6.2%) had post-closure leakage with duct diameters of 3 mm and 4 mm. Both were type A ducts, and all patients were monitored for residual shunts, with no complications such as hemolysis or infection reported. Long-term follow-up showed no ductus recanalization or stenosis among those with complete occlusion. The average follow-up duration was 105.8 months (SD ± 55). Success rates were high: 97.5% in Group 1, 100% in Group 2, and 99% in Group 3, with an overall rate of 97.5%. Follow-up evaluations were conducted immediately post-procedure, the following day, at six months, twelve months, and annually.

No cases of obstruction or significant gradients were found, and long-term monitoring showed no major complications such as permanent shunts, hemolysis, or infective endocarditis.

Discussion

Isolated PDA occurs in full-term infants at rates of 0.03% to 0.08% and is more common in females. In our study, 203 patients (63.4%) were female, compared to 117 males (36.4%), resulting in a female-to-male ratio of 1.73. We noted that PDA prevalence was higher among females across all device groups, consistent with the existing literature (2). Since 1938, surgical interventions have been essential in PDA treatment, with the transcatheter method gaining traction since 1967 (4). While Gianturco coils were commonly used in the 2000s, their popularity has declined due to the introduction of newer devices and the risks associated with multiple coils and embolization. As a result, the long-term outcomes of coils have been less studied (9-11). In our research, we achieved a 97.5% success rate in transcatheter PDA closure using coils, aligning with previous success rates of 89% in Germany, 90.5% by Galal (12), and 94.6% in a series involving 243 patients (11). The ADO-I device is shaped like a mushroom, while the ADO-II resembles an umbrella (13). Both devices automatically adopt their intended forms due to an innovative memory feature. The ADO-I has a larger disc on the aortic side for secure fixation, while the ADO-II features equally sized discs at both ends with a narrower waist in the middle. These devices effectively close PDA and have shown strong results. In a study of 29 patients under one year of age, 26 (89.6%) achieved successful duct closure with an ADO device. The complete closure rates were 73.1% immediately after the procedure, rising to 84.6% after 24 hours and 96.1% by the third month (14). Notably, the ADO device has a complete closure rate exceeding 98% at six months and very low complication rates (15). ADO devices have been used in our clinic since 2008. Our study achieved a 100% success rate in transcatheter PDA closure with ADO-I, with no residual shunts detected in any patients. This underscores the high success and low residue rates of ADO devices compared to coils (12-15). Our procedure success rates were 97.5% in Group I, 100% in Group II, and 99% in Group III. The ADO group consistently showed higher success rates than the coil group, with no significant difference between ADO-I and ADO-II ($p>0.05$). Small-diameter and long PDA devices may not be suitable for all patients, so device selection should depend on the PDA type and size. Most patients in our study had small PDAs, and some underwent closure before ADO devices became widely available, leading to more experience with coil closures.

Device embolization is a significant concern, with rates varying from 0% to 6% (16-18). Coil closures have a higher risk of embolization (about 4%) compared to ADO devices (less than 1%) (16-18). In our study, embolization was noted in four coil closure patients and one patient with an ADO-II device, requiring surgical intervention to remove the embolized devices. Our clinic's transition to ADO devices post-2008 and the cost-effectiveness of the closure procedure influenced the decision for surgery. Device embolization during the release of ADO-I usually occurs in the pulmonary artery but can extend to systemic circulation. A study of 209 patients found three cases of embolization, while our study reported none with ADO-I (6). However, caution is advised when using ADO-I in young children under 5 kg, as it may cause obstruction in the pulmonary artery or aorta (14). The ADO-II device is designed for safe use in infants and offers anterior and posterior placement options (19,20). In our study, ADO-II was successfully used in 107 patients (33.4%) with a 99% success rate for transcatheter PDA closure. Overall, the coil embolization rate was only 1.9%, with none in Group II and just one case (0.93%) in Group III. After percutaneous closure of a PDA, there is a risk that closure devices may protrude into the aorta or cause stenosis in the left pulmonary artery, potentially related to the retention disc or the use of larger coils (19-21). In our study, echocardiographic Doppler evaluations before discharge showed no stenosis in any patients. In a study of 62 patients with a median age of 1.2 years who underwent transcatheter PDA closure with ADO-II, the residual shunt rate was 5% immediately post-procedure, dropping to 0% at one year and in long-term follow-ups (22). Our findings showed a 1% residual shunt rate in the coil group the day

after closure, while no residual shunt was detected in the ADO-I and ADO-II groups. By the one-year follow-up, all patients with residual shunts in the coil group had closed spontaneously.

Study Limitations

Our literature review highlights the limited use of the ADO-I device for PDA closure, with only 12 patients in our study receiving this treatment. The most common ductal structure among these patients was Type B, which is more challenging to close via transcatheter methods. We propose that ADO-I devices may be the preferred choice for Type B ductus closures. While studies are limited, Faella and Hijazi (23) suggested that ADO devices could be effective for cases of window-type ductus. However, we recommend multicenter studies with larger patient cohorts to confirm our findings. A key limitation of this study was the preference for coils and ADO-II devices for occluding PDA up to 3 mm in diameter, resulting in varying duct sizes among the participants. The small sample size and reliance on retrospective data collection further restrict our findings, particularly due to the limited number of patients with ADO-I devices, which affected statistical comparisons. Larger, multicenter studies with prospective data collection would provide stronger evidence for using these devices in pediatric patients.

Conclusion

Recent advancements have improved the closure of PDA via transcatheter procedures, achieving high success rates. Patient selection is critical, requiring careful consideration of age, weight, duct dimensions, and device size. Although the use of coils has decreased, they remain suitable for smaller duct diameters. ADO-I devices work best for large PDAs, while ADO-II devices are recommended for small to medium PDAs with shorter ducts. ADO-I devices are also ideal for Type B ducts.

Ethics

Ethics Committee Approval: This study was approved by the Medical Research Ethics Committee of Ege University (approval no.: 24-3T/74, date: 07.03.2024).

Informed Consent: Informed consent was obtained.

Footnotes

Authorship Contributions

Surgical and Medical Practices: Ş.Ş.Ö., E.D., F.E., M.B.B., G.K.K., M.Y., Concept: Ş.Ş.Ö., F.E., B.B.A., Z.Ü.T., Design: Ş.Ş.Ö., E.D., F.E., M.B.B., G.K.K., M.Y., B.B.A., B.K.B., Z.Ü.T., R.E.L., Data Collection or Processing: Ş.Ş.Ö., F.E., M.B.B., G.K.K., M.Y., B.K.B., Z.Ü.T., R.E.L., Analysis or Interpretation: Ş.Ş.Ö., E.D., G.K.K., M.Y., B.K.B., Z.Ü.T., R.E.L., Literature Search: Ş.Ş.Ö., B.B.A., B.K.B., Z.Ü.T., R.E.L., Writing: Ş.Ş.Ö., F.E.

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

Financial Disclosure: The authors declared that this study received no financial support.

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J Pediatr Res 2024;11(4):212-219
DOI: 10.4274/jpr.galenos.2024.89137

Original Article

Demographic Features, Clinical, and Laboratory Findings of Partial and Selective IgA Deficiency in Children

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ABSTRACT

Aim: Immunoglobulin A deficiency (IgAD), which is the most common primary antibody deficiency, can cause clinical problems due to significant infections and associated diseases, while some individuals with IgAD remain symptomless throughout their lives. This study evaluated the demographic features, clinical, and laboratory findings for those patients with selective and partial IgAD.

Materials and Methods: A retrospective study was conducted at İstanbul University-Cerrahpaşa, Cerrahpaşa Faculty of Medicine, Clinical Immunology, and Allergy Outpatient Clinic. This study included 149 children, 122 diagnosed with selective and 27 diagnosed with partial IgAD.

Results: The median age at diagnosis was 6 years, and the majority of the patients (55.0%) were male. Nine patients transitioned from selective to partial IgAD, while four patients switched from partial to selective IgAD. The majority of patients experienced infections (56.3%) and allergies (47.7%). Autoimmune diseases were present in 14.1% of the study group; thyroiditis was the most common. Immunoglobulin G (IgG) subgroup deficiencies were detected in 20.2% of 84 patients who were examined. B-cell subpopulation analysis was carried out in 22 patients, revealing differentiation abnormalities in 18.1%. Two of these patients were siblings; one had low CD27+IgD-class-switched memory B-cells.

Conclusion: This study revealed that infections were the most common concern, but the frequencies of allergic manifestations and autoimmunity were also significant. While studies on B lymphocyte subgroup analysis continue to gain importance, the presence of patients with defects was observed in this study. Following an IgAD diagnosis, patients should undergo close immunological and clinical monitoring.

Keywords: IgA deficiency, immunodeficiency, autoimmunity, allergies, B lymphocyte

Introduction

Immunoglobulin A deficiency (IgAD) occurs due to a defect in immunoglobulin A (IgA) production, although other immunoglobulin isotypes can be produced by B-cells (1). It is the most common primary antibody deficiency (2,3). Although the pathogenesis has not been explained yet, defects of B-cells in terminal differentiation or transformation into IgA-producing plasma cells have been implicated (3,4). IgA-producing plasma cells in the peripheral circulation are reduced, and immature B-cells are increased (2,4). Furthermore, a decline in Iα germline transcription prior to class transformation has been identified as the underlying factor for selective IgAD. It is also proposed that partial IgAD arises due to the inhibition of B-cell development following class transition (5). Another perspective suggests that heightened depletion of B-cell subsets may be the underlying cause of the deficiency (4).

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Received: 17.07.2024 **Accepted:** 09.09.2024

The primary antibody of the mucosal immune system is secretory IgA, and if deficient, microorganisms, aeroallergens, and food-borne allergens easily penetrate the mucosal barrier and enter the body (3). Therefore, the common complaints are sinopulmonary, gastrointestinal system infections, allergies, and autoimmune disorders (6). Most individuals with impaired IgA production are asymptomatic (approximately two-thirds); these patients can be diagnosed via random immunoglobulin examinations, while the remaining one-third progress with clinical symptoms (7).

Selective and partial IgAD generally have a favorable prognosis, and it is possible for their immunoglobulin levels to rise during the course of their follow-up (2,8). However, chronic allergy complaints, autoimmune diseases, and accompanying immunological disorders closely relate to the course of this disease and the severity of infections. Assessing the different subgroups of B lymphocytes in the development of diseases has become increasingly important in recent years. Class-switched memory B lymphocytes have been demonstrated to be a crucial biomarker for clinical prognosis in patients (7).

The objective of our study was to analyze the demographic characteristics of those patients diagnosed with selective or partial IgAD. We aimed to identify their complaints upon admission, clinical findings, associated diseases, infections, complications, and any differences between the two groups by reviewing their laboratory data. This study aimed to present clinical experience of over 30 years and to enhance awareness regarding this disease.

Materials and Methods

A retrospective cohort study was conducted in the Cerrahpaşa Faculty of Medicine, Department of Pediatrics, Clinical Immunology and Allergy Outpatients' Clinic, İstanbul University-Cerrahpaşa, İstanbul, Turkey. Those patients diagnosed with IgAD between the ages of 0 and 18 during the prior 30 years, beginning in 1989, were assessed. Demographic characteristics, underlying medical conditions, clinical findings and laboratory characteristics, medical treatment, complications, and prognosis were collected from the medical records. Autoimmune diseases, allergies, other accompanying diseases, and previous infections were documented during the follow-up period.

Definitions

The diagnosis of selective IgAD was confirmed over four years of age by an IgA level below 7 mg/dL with normal immunoglobulin M (IgM) and IgG levels, excluding other causes of hypogammaglobulinemia (2). Partial IgAD was defined as an IgA level above 7 mg/dL but below the expected 2 standard deviations (SD) for age (2).

The serum levels of IgG, IgA, IgM, and immunoglobulin E (IgE) in the patients were determined using the nephelometric method. These values were categorized as either low or high based on SD and age. IgG subgroup deficiency is defined in patients over seven years of age with at least one of the IgG subgroups below the 5th percentile. Immunoglobulin and IgG subgroups were analyzed with reference to the study by Aksu et al. (9).

Lymphocyte subgroups (CD45, CD4, CD8, CD19, CD20, CD16-56) were recorded, and those below 2 SD of normal levels for age were classified as low. These percentages and values were examined taking the research by Ikincioğulları et al. (10) as the reference. A flow cytometer was used to assess the results of B lymphocyte subgroups (CD19, CD19+CD27+, CD27-IgD+, CD27+IgD+, CD27+IgD-, CD27-IgD-, CD38highCD24high). These results were compared to the values of B lymphocyte subgroups determined by Duchamp et al. (11).

Ethics

Cerrahpaşa Faculty of Medicine, İstanbul University-Cerrahpaşa Clinical Research Ethics Committee approved this study (date: 15.12.2018, no: 29430533-90399).

Statistical Analysis

Statistical analysis of the research data was carried out with the SPSS v21 (IBM Corp., Armonk, NY, USA) package program. Descriptive data, frequencies, and percentages are given for categorical variables and compared by the Pearson chi-square test. Descriptive analyses are given using means and SD for normally distributed variables, and Student's t-test was used to compare two independent sample groups; since continuous variables did not meet the normal distribution conditions, they are given using median, minimum and maximum and evaluated using non-parametric tests (the Mann-Whitney U test). Statistical significance was accepted as $p<0.05$.

Results

There were a total of 149 patients in the study cohort, consisting of 122 selective IgAD and 27 partial IgAD. The majority of the patients, eighty-two, were male (55.0%). Nine patients (6.0%) were diagnosed with selective IgAD due to an IgA level below 7 mg/dL at admission. However, during follow-up, their IgA rose roses but still were 2 SD below their age expected levels, and so they subsequently

classified as partial IgAD. Despite being below 2 SD by age at diagnosis, four patients (2.6%) transitioned from partial IgAD to selective IgAD as a result of their IgA values decreasing below 7 mg/dL. Table I illustrates the demographic characteristics of the patients.

Two families (2.7%) had a history of primary immunodeficiency, and the rate of consanguineous marriage was found to be 11.4% of the study group. There were twins with selective IgAD and siblings with selective and partial IgAD. The median age of the patients at the onset of symptoms was 3 years, and the median age at hospital admission was 6 years (Table I). At the time of admission, the age divisions of the patients were as follows: 25.5% were between the ages of 0 up to 4 years, 39.6% were between the ages of 4 up to 8 years, 22.1% were between the ages of 8 up to 12 years, and 12.8% were 12 years of age or older. The mean follow-up period for our patients was 4.99±4.3 years. Some of our patients were monitored until the age of four, while others were monitored until the age of 22. Twenty-two patients (14.7%) had underlying diseases.

Table I. The demographic and clinical characteristics of the study population

Characteristics	**n (%)**
Selective IgA deficiency	122 (81.8)
Partial IgA deficiency	27 (18.1)
Male	82 (55.0)
Consanguineous marriage	17 (11.4)
Primer immune deficiency in family	4 (2.6)
Accompanying autoimmune diseases	21 (14.1)
-Autoimmune thyroiditis	9 (6.0)
-Juvenile idiopathic arthritis	2 (1.3)
-Celiac disease	2 (1.3)
-Type 1 diabetes	2 (1.3)
-Inflammatory bowel disease	2 (0.6)
-Systemic lupus erythematosus	2 (0.6)
-Vasculitis	1 (0.6)
-Vitiligo	1 (0.6)
-Myasthenia	1 (0.6)
-Psoriasis	1 (0.6)
-Mixed connective tissue disease	1 (0.6)
Other underlying diseases	22 (14.7)
Frequent infections	84 (57.3)
Allergies	71 (47.7)
Age at admission (median, min.-max.)	6 years (6 months-17 years)
Age at onset of symptoms (median)	3 years (6 months-16 years)

Ig: Immunoglobulin, min.-max.: Minimum-maximum

The most common complaint at admission was frequent infections (n=84, 56.3%), defined as eight or more infection episodes yearly. Patients predominantly suffered from frequent upper respiratory tract infections (n=135, 90.6%), followed by 56 (37.6%) with frequent fever, 45 (30.2%) with lower respiratory tract infections (LRTIs), 24 (16.1%) with otitis, 43 (28.9%) with sinusitis, 24 (16.1%) with urinary tract infections, and 19 (12.8%) with gastroenteritis. Eleven (7.4%) patients had been hospitalized due to infectious diseases. Bronchiectasis developed in 2 (1.3%) of the patients, no other underlying immunodeficiencies were detected in either of them and it was thought to have developed following a severe LRTI. When the selective and partial IgAD groups were compared in terms of previous infections, the rate of frequent sinusitis was significantly higher in the selective IgAD group (32.7% vs. 11.1%, p=0.02). It was determined that ten patients (6.7%) used prophylactic antibiotics during their follow-up.

Allergy

Allergy was seen in 71 (47.7%) patients with IgAD; 49 (32.8%) had one allergic complaint, and 22 (14.7%) had multiple. Asthma was the most common allergic manifestation in 52 patients (34.8%) followed by allergic rhinitis diagnosed in 25 (16.8%), allergic skin rash in 16 (10.7%), and food allergy in 6 (4.0%). There was no significant difference in the prevalence of allergic illnesses between the selective and partial IgAD groups (p=0.36). Among those patients exhibiting hypersensitivity (n=71), 69.0% reported using medication. Specifically, 38 (53.5%) used leukotriene receptor antagonists, 22 (30.9%) used inhaler steroids, 24 (33.8%) used bronchodilators, 13 (18.3%) used antihistaminic drugs, and 14 (19.7%) used nasal steroids.

Autoimmune Diseases

Autoimmune diseases were present in 21 (14.1%) of all patients, with autoimmune thyroiditis being the most prevalent (n=9, 6%) (Table I). Three patients were diagnosed with more than one autoimmune disease. During the diagnostic procedures for autoimmune diseases, nine patients (6.0%) were diagnosed with IgAD. The appropriate diagnosis, treatment, and follow-up processes for autoimmune diseases were carried out by the relevant

departments. There was no significant difference between selective and partial IgAD for accompanying autoimmune diseases (p=0.46).

Immunoglobulins and Subgroups

The median value of IgG was significantly higher in the selective IgAD group (p=0.01). There was no significant difference between the median IgM values (p=0.77) (Table II). It was observed that the IgM level was elevated in 15 patients (10.0%) at the time of first admission.

IgG Subgroup Deficiency

IgG subgroups were studied in 84 patients, and low values were detected in 17 (20.2%) of them. Eight patients (9.5%) had low IgG4 values, four (4.7%) had low IgG3 values, two (2.3%) had low IgG2 and IgG4 values, one (1.2%) had low IgG1 values, one (1.2%) had low IgG3 and IgG4 values, and one (1.2%) had low IgG1 and IgG4 values. All of these patients were in the selective IgAD group. There was no significant difference in absolute values of the IgG subgroups between the patient groups (Table II).

Lymphocyte Subgroups

There was no significant difference between the patient groups for lymphocyte subgroup percentages. It was observed that the CD4/CD8 ratio was reversed in 12 patients (8.0%). In comparison to the reference values, the percentages of lymphocyte subgroups were within the normal range (Table III).

B lymphocyte Subgroups

Four patients (18.1%) exhibited B-cell differentiation defects when compared with the reference values during the examination of 22 patients. There was no significant difference between the patient groups for the mean percentages measured in 22 patients (Table IV).

In two patients, the total number of memory B-cells was decreased, while the number of naive B-cells was increased. The number of class-switched memory B-cells was decreased in one of these patients, while the number of non-class-switched B-cells was decreased in the other. A third patient exhibited a decrease in non-class switched memory B-cells, while a fourth patient exhibited an increase in naive B-cells. The characteristics of these patients are detailed in Table V.

Table II. IgG, IgM, and IgG subgroup absolute values of selective and partial IgA deficiency patients

	Selective IgA deficiency (median, min.-max., mg/dL)	Partial IgA deficiency (median, min.-max., mg/dL)	p value
IgG	1,420 (149-2,930)	1,080 (464-3,549)	**0.017***
IgM	116 (41-846)	110 (51-228)	0.771
IgG1*	1,035 (364-2,190)	834 (593-2,190)	0.296
IgG2*	307 (37-934)	227 (97-1,130)	0.377
IgG3*	45.4 (0.5-139)	45.6 (17-101)	0.980
IgG4*	31 (8.2-368)	35.2 (8.2-301)	0.352

*: IgG1, IgG2, IgG3, and IgG4 were determined in 84 patients, Ig: Immunoglobulin, min.-max.: Minimum-maximum

Table III. Lymphocyte subgroup percentages of selective and partial IgA deficiency patients*

	Selective IgA deficiency (median, min.-max., %)	Partial IgA deficiency (median, min.-max., %)	p value
CD45	97.4 (81.0-100)	98.1 (56.0-100)	0.569
CD3	70.0 (57.3-82.0)	68.9 (52.0-88.0)	0.754
CD4	40.0 (17.2-69.0)	40.0 (31.3-69.0)	0.588
CD8	30.7 (18.0-50.0)	26.6 (6.0-38.0)	0.310
CD19	15.1 (6.4-25.0)	19.0 (7.4-85.0)	0.156
CD20	18.0 (3.0-29.0)	17.1 (11.4-35.0)	0.440
CD16-56	9.9 (4.0-21.2)	9.5 (4.0-32.4)	0.694

*: Lymphocyte subgroups were determined in 65 patients, Ig: Immunoglobulin, min.-max.: Minimum-maximum

Table IV. B Lymphocyte subgroup percentages of selective and partial IgA deficiency patients*

	Selective IgA deficiency (n=17)	Partial IgA deficiency (n=5)	p
CD19 + B-cells	13.3±3.7	15.2±4.1	0.401
CD19 + CD27 + B memory cells	21.0±10.8	24.7±11.5	0.359
CD27- IGD+ naive B-cells	73.1±12.5	67.6±12.9	0.283
CD27+IGD+ non-class switched B memory	9.9±6.3	9.1±3.5	0.940
CD27+IGD- class-switched B memory	11.2±6.7	14.9±7.4	0.359
CD38 high CD24 high transitional B-cells	2.5±1.8	2.5±1.2	0.649

*: B- Lymphocyte subgroups were determined in 22 patients, Ig: Immunoglobulin

Table V. Characteristics of patients with B-cell subgroup anomalies

Patient no	Subgroup features	Patient characteristics
1	Memory B-cells decreased Naive B-cells increased Class-switched memory B-cells decreased	Partial IgA deficiency, selective IgA deficiency in her sister Allergic rhinitis and asthma IgM and IgE increased CD4/CD8 ratio reversed
2	Memory B-cells decreased Naive B-cells increased Non-class switched memory B-cells decreased	Selective IgA deficiency IVIG therapy for transient hypogammaglobulinemia IgG3 and IgG4 deficiency CD4/CD8 ratio reversed Diarrhea attacks, persistent sinopulmonary infections, and otitis media
3	Non-class switched memory B-cells decreased	Selective IgA deficiency Persistent sinopulmonary infections and otitis media
4	Naive B-cells increased	Selective IgA deficiency Persistent sinopulmonary infections and asthma

Ig: Immunoglobulin, IVIG: Intravenous immunoglobulin

Discussion

Immunoglobulin A deficiencies have been examined from different perspectives via clinical findings, accompanying diseases, and immunological laboratory values, however, the pathogenesis of IgAD has not yet been significantly resolved. Our study observed that some patients diagnosed with partial IgAD could switch to selective IgAD, while others in the selective group could switch to partial deficiency. Moschese et al. (12) also observed that there were transitions between groups, as in our study, and they also reported that normalization of IgA levels was significantly more common in partial IgAD than in selective IgAD. Plebani et al. (13) observed that serum IgA can rise to normal levels in partial deficiencies but did not reach the normal range in severe deficiencies. A substantial number of patients demonstrated reversals in a comprehensive study conducted in Sweden, despite the fact that a diagnosis of IgAD was made after the age of 4 years (14). It is important to monitor these transitions and the potential for reversals in the risk assessment and follow-up of these patients.

Karaca et al. (15) studied the families of patients with common variable immunodeficiency (CVID) and IgAD in Turkey and observed that 33.6% of the cases were familial, 19.1% had low immunoglobulin levels, and the familial cases were more severe. Two familial cases were identified in the course of our research. The fact that two of our patients were siblings and both were diagnosed with IgAD suggests that individuals in a family with frequent infections should be screened for immunodeficiencies. The most significant risk factor for the development of IgAD and CVID is a positive family history and it is recommended that the families of IgAD and CVID patients undergo routine screening (15).

This study observed that the patients' complaints could begin over a wide age range, and most applied to a physician before 4 years of age. The period between the onset of symptoms and their application was approximately 3 years. This interval shows that awareness is not enough for both families and physicians. During the follow-up, the majority of their complaints consisted of infections. LRTIs and hospitalization rates reflect the clinical severity of these patients. There is a statistical difference in the frequency of sinusitis, although there were more cases of LRTI, bronchiectasis, and hospitalization in the selective IgAD without a significant statistical difference but with a

clinical significance. Moschese et al. (12) reported infections of the respiratory/gastrointestinal tract were the most common clinical manifestations in both selective IgAD (53%), and partial IgAD (64%). Our results demonstrate that both selective and partial IgAD patients were mostly struggling with recurrent infections, as previously reported. Additionally, selective IgAD patients exhibited slightly elevated infection rates.

Hypersensitivity was present in 47.7% of our patients, and most of them used medication (69.0%). Aytekin et al. (1) found that allergies (43.2%) were the second most common complaint following infections in patients with IgAD. Cinicola et al. (16) found the prevalence of allergy in the pediatric IgA deficiency cohort was 34%, while Shkalim et al. (17) found that 31.7% of patients with IgAD had allergic disorders, with asthma being the most prevalent, followed by allergic rhinitis, which aligns with our own findings. Abo Ali et al. (18) also revealed that the percentage of IgAD in asthmatic patients was 56%. This highlights the high frequency of allergy in IgAD patients and the necessity of vigilant monitoring of these individuals for allergic manifestations during both the diagnosis and follow-up phases.

IgAD and IgG subgroup deficiency can be seen together, causing more severe infections (8). Within this study, a total of 17 out of 84 patients with selective IGAD and IgG subgroup deficiency, representing 20.2% of the sample, reported recurring infection symptoms along with the presence of autoimmune disorders and allergies. Karaca et al. (19) observed in the IgG subgroup deficient pediatric patients that IgG3 deficiency was the most common, while they had recurrent infections, and that 30-40% recovered after six years of age. The fact that all of the patients with complete IgG subclass deficiency belonged to the selective IgAD patient group and that, as previously mentioned, the rates of serious infections were found to be higher in the selective IgAD group highlights the need for close monitoring of the selective IgAD group as they are considered to be at higher risk.

Autoimmunity has been the subject of extensive research and discussion in patients with IgAD in recent years. The association between IgAD and autoimmunity may be explained by the relationship between IgAD and abnormal T-cell regulation, particularly in regulatory T-cells and reduced switching memory B-cells (20). Autoimmunity was present in 14.1% of the participants in our investigation. Depending on the country, the study center, and the number of patients involved, the reported rates varied both in Turkey and in global studies. Abolhassani et al. (20) determined that the autoimmunity rate was 29.8% in their study. In their review, Vosughimotlagh et al. (21) revealed that the prevalence of autoimmunity ranged from 4.2% to 39.2%. Aytekin et al. (1) investigated children who were diagnosed with selective IgAD and an autoimmune disease was reported to be present in 17% of children. However, they demonstrated that 31% of these patients had positive autoantibodies, and only a portion of them had an autoimmune disease. In Sweden, Ludvigsson et al. (22) conducted a national study in which 2,100 patients with IgAD were screened, and the results indicated that the rates of autoimmune diseases in patients from the age of four to adulthood were significantly higher than in society.

Celiac disease was identified as the most prevalent autoimmune disease in a meta-analysis (21). IgAD may also be associated with autoimmune thyroiditis, celiac disease, juvenile idiopathic arthritis, idiopathic thrombocytopenic purpura, hemolytic anemia, psoriasis, inflammatory bowel disease, and systemic lupus erythematosus (23). According to Erkoçoğlu et al. (24), the most prevalent autoimmune disorder in children with selective IgAD was celiac disease (9.9%), followed by Type 1 diabetes mellitus (DM) (3.7%) and thyroiditis (2.5%). The most prevalent autoimmune condition among our patients was autoimmune thyroiditis, which affected 6.0% of individuals. This was followed by celiac disease, type 1 DM, inflammatory bowel disease, and juvenile idiopathic arthritis, which each affected 1.3% of our patients. Although the high occurrence of celiac disease has been extensively documented in studies, it is important to highlight that autoimmune thyroiditis was the predominant condition in our study, highlighting the necessity of diagnosing and subsequently monitoring these individuals.

IgM levels were above the normal limits for age in 15 patients (10.0%) at first admission, two of whom had autoimmunity. It is thought that this elevation occurred to compensate for the decrease in secretory IgA (4). When lymphocyte subgroups were assessed separately, it was noted that the proportion of B-cells and natural killer (NK) cells were within the normal ranges. In certain patients, the CD4/CD8 ratio was reversed, despite the fact that T-cells were within normal limits. Nechvatalova et al. (6) similarly reported that the number and ratio of CD4 + T lymphocytes were decreased and CD8 + T lymphocytes were increased in IgAD patients.

Our study showed abnormalities with differentiation in B-cell subgroups in 18.1% (4 out of 22) of the patients

investigated. Aghamohammadi et al. (7) put forward that those patients with problems in these stages of maturation, especially those with low class-switched memory B-cells, may have a more severe course, the development of autoimmunity and immunodeficiency (especially CVID) by evaluating the subgroups of B lymphocytes. B lymphocyte subgroup analysis is an essential biomarker in clinical prognosis according to recent studies. Bukowska-Straková et al. (25) showed that low CD27+IgD-class-switched memory B-cells are common in patients with CVID and IgAD. Nechvatalova et al. (6) also showed that in IgAD patients class-switched memory B-cells and IgM-plasmablasts were decreased, $CD21^{low}CD38^{low}$ B-cells were increased, and these anomalies were similar to patients with CVID. Celiksoy and Yildiran (26) studied patients with antibody deficiency and found that naive B-cells were increased in patients with CVID and IgAD, similar to our patients 2 and 4. Additionally, both class-switched and non-class-switched memory cells were decreased in CVID, and total memory cells and non-class-switched memory cells were decreased in IgAD. These studies showed that although it is more common in CVID, CD27+ IgD- class-switched memory B-cells are decreased in IgAD, similar to our patient 1. Given that patients 1 and 2 were siblings and considering the depletion of CD27+IgD-class-switched in patient 1 and the reduction of non-class-switched memory B-cells in patients 2 and 3, it is recommended that they be closely monitored and evaluated prospectively regarding CVID. This may be due to a common genetic predisposition, and this situation can guide the clinical prognosis and prediction of CVID (6,26).

Conclusion

Upon analyzing and comparing both selective and partial IgAD, this study revealed that infections were the predominant issue, however allergy symptoms and autoimmunity also exhibited notable frequencies. Although the frequency of infection was very high in both groups and there was no difference for accompanying allergic complaints and autoimmune diseases, in the selective IgAD group, sinusitis, LRTIs, hospitalizations and bronchiectasis occurred more frequently, and IgG subgroup deficiencies were only seen in the group with selective IgAD, so it can be concluded that selective IgAD must be monitored more closely and changes in patients' IgA levels are important in determining risk. As research on the study of B lymphocyte subgroups becomes more significant, it has been noted that several individuals who were examined had problems. Ultimately, patients diagnosed with IgAD should undergo extensive immunological and clinical monitoring during their follow-up.

Ethics

Ethics Committee Approval: Cerrahpaşa Faculty of Medicine, İstanbul University-Cerrahpaşa Clinical Research Ethics Committee approved this study (date: 15.12.2018, approval no.: 29430533-90399).

Informed Consent: Retrospective study.

Footnotes

Authorship Contributions

Surgical and Medical Practices: A.A., Concept: A.A., H.Ç., Y.C., Design: A.A., H.Ç., Data Collection or Processing: A.A., Analysis or Interpretation: A.A., Y.C., Literature Search: A.A., Writing: A.A., H.Ç., Y.C.

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

Financial Disclosure: The authors declared that this study received no financial support.

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J Pediatr Res 2024;11(4):220-224
DOI: 10.4274/jpr.galenos.2024.79989

Original Article

Hot Water Epilepsy in Children: A Rare Form of Reflex Epilepsy

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ABSTRACT

Aim: We evaluated the clinical and electroencephalography (EEG) characteristics, treatments, and outcomes of children with hot-water epilepsy (HWE), a specific type of reflex epilepsy.

Materials and Methods: This retrospective study included 11 children who were followed-up for HWE in a pediatric neurology department between 2005 and 2022.

Results: Eight children (73%) were boys and three (27%) were girls. The mean age was 60.7±30.8 months (range 11-110) and the mean age at seizure onset was 57±31.7 months (range 11-108). The mean follow-up duration was 20 months (range 10-32 months). The seizure type was identified as focal onset impaired awareness in six cases (54%) and generalized tonic-clonic seizures in five patients (46%). Four (36%) patients experienced spontaneous seizures. Interictal EEG was abnormal in two patients (18%). Four patients with spontaneous seizures were initially recommended bathing with lukewarm water and continuous anti-seizure medications. Three patients with reflex seizures benefited from lukewarm bathing and achieved seizure control. Two patients with uncontrolled reflex seizures were seizure-free after clobazam prophylaxis. Two patients who were unresponsive to lukewarm bathing and clobazam were started on continuous anti-seizure medications. In total, six patients were on continuous anti-seizure medications.

Conclusion: Despite its benign nature, HWE should be identified and appropriately treated due to the risk of spontaneous seizures. It is also important to determine the triggering factors so that appropriate bathing with lukewarm water and intermittent clobazam prophylaxis can be initiated. Spontaneous seizures require anti-seizure medications.

Keywords: Hot water epilepsy, children, clobazam

Introduction

Seizures can be precipitated by certain stimuli, including hot water and visual stimuli, in approximately 5% of epilepsy patients and these are defined as reflex epilepsies (1). Hot water epilepsy (HWE) is a specific type of reflex epilepsy which occurs with the pouring of hot water over the head. It was first described in 1945 in New Zealand (2). It is most common in South India, and is reported to constitute 3.6-3.9% of epileptic seizures (3). Turkey is one of the countries where HWE is most frequently reported (4-7). Its etiopathogenesis is not clear, but genetic and environmental factors and bathing habits with hot water have been implicated (8).

In this study, we evaluated the clinical and electroencephalographic (EEG) characteristics, treatments, and outcomes of children with HWE.

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Received: 02.07.2024 **Accepted:** 15.09.2024

Materials and Methods

This study included 11 children who were followed up for HWE in the pediatric neurology department between 2005 and 2022. Data were retrospectively retrieved from the hospital and patient records, including patient characteristics, age at seizure onset, seizure semiology, the presence of spontaneous seizures, the presence of febrile seizures, family history of epilepsy, developmental status, duration of follow-up, response to modification of bathing habits, and antiepileptic treatment. Neurological and cardiac examinations, laboratory results, imaging, and EEG findings were evaluated. The history of seizures was based on the accounts of family members. This study was approved by the Institutional Review Board of Koç University (approval no.: 2023.077.IRB1.025, date: 09.03.2023). The analysis and reporting of the results complied with the strengthening the reporting of observational studies in epidemiology checklist.

Statistical Analysis

Data were analyzed using descriptive statistics and were expressed as number and percentages and mean±standard deviation, where appropriate.

Results

Eight of the children (73%) were boys and 3 (27%) were girls, with a male-to-female ratio of 2.6. The mean age was 60.7±30.8 months (range 11-110 months) and the mean age of seizure onset was 57±31.7 months (range 11-108 months). None of the patients had any history of febrile convulsions. Three patients (37%) had a family history of HWE, two of whom were siblings. Two (18%) patients had a family history of febrile convulsions. The mean follow-up duration was 20 months (range 10-32 months). Seven patients (64%) only had reflex seizures induced by hot water and four patients (36%) also had spontaneous seizures, which appeared after an interval of six to 12 months.

Seizure type was identified as seizures characterized by focal onset impaired awareness in six cases (54%) and generalized tonic-clonic seizures in five patients (46%), followed by post-ictal headache in one patient. All of the patients had normal development and neurological examination findings.

Brain magnetic resonance imaging (MRI) was unremarkable in all patients. Interictal EEG recordings were normal in nine patients (82%). The abnormal EEG findings in two patients (18%) included sharp wave activity in the temporal part of the right hemisphere in one patient

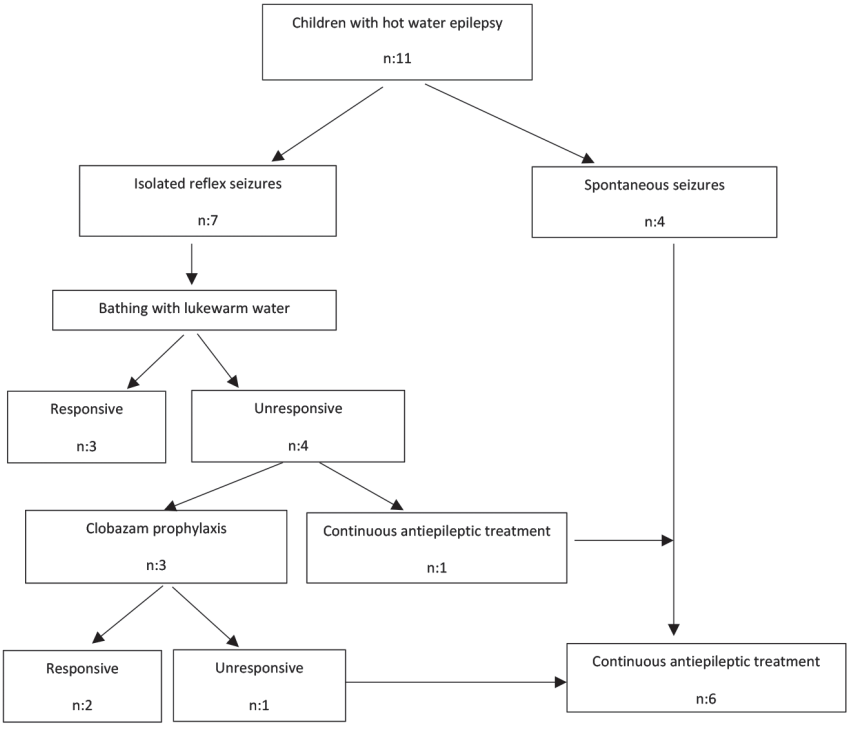

Figure 1. Flow chart of management of patients with hot water epilepsy

(Patient 11) and slow-wave discharges in the left temporo-occipital region in the other (Patient 7).

Treatments

Those patients with reflex seizures and spontaneous seizures were initially recommended to bathe with lukewarm water (Figure 1). Continuous antiepileptic treatment was initiated in those patients with spontaneous seizures. Of the seven patients with reflex seizures only, three benefited from lukewarm bathing and had seizure control. Three patients with uncontrolled reflex seizures received clobazam prophylaxis approximately one hour prior to bathing, of whom two had seizure control. Two patients who initially had reflex seizures but were unresponsive to lukewarm bathing or clobazam were started on continuous antiepileptic treatment. In total, six patients were on continuous anti-seizure medications.

Anti-seizure medications included carbamazepine (n=4), levetiracetam (n=1), and valproic acid (n=1) (Table I).

One patient who received carbamazepine for spontaneous seizures also received clobazam prophylaxis due to the persistence of hot water-induced seizures. Five patients underwent continuous antiepileptic treatment. One patient (Patient 6) who discontinued carbamazepine after four months of antiepileptic treatment remained seizure-free during follow-up without any anti-seizure medications (Figure 1).

Table I. Patients' characteristics

Patient no	Gender	Age of onset (months)	Age at presentation (months)	Family history	Seizure semiology	Presence of spontaneous seizures	EEG	Follow-up (months)
1	F	24	28	HWE in a sibling	Generalized	+	Normal	24
2	M	108	108	No	Focal onset impaired awareness	+	Normal	16
3	M	36	48	No	Generalized	-	Normal	18
4	M	42	48	HWE in a sibling	Generalized	-	Normal	30
5	F	62	64	SSE	Focal onset impaired awareness	+	Normal	17
6	M	106	110	Febrile convulsion	Generalized	-	Normal	32
7	M	11	11	No	Focal onset impaired awareness	-	Abnormal	20
8	M	46	48	No	Focal onset impaired awareness	+	Normal	10
9	F	60	66	No	Focal onset impaired awareness	-	Normal	24
10	M	46	50	Febrile convulsion	Generalized	-	Normal	14
11	M	87	87	No	Focal onset impaired awareness	-	Abnormal	15

EEG: Electroencephalography, HWE: Hot-water epilepsy, CBZ: Carbamazepine, CLB: Clobazam

Table I. Continued

Patient no	Time interval between spontaneous and reflex seizures (months)	Response to lukewarm bathing	Antiepileptic medications	Prognosis
1	8	-	VP	A single spontaneous seizure during non-adherence.
2	10	-	CBZ, CLB	Seizure control following addition of intermittent CLB to CBZ for ongoing hot water seizure
3		+	-	Seizure-free
4		+	-	Seizure-free
5	12	-	CBZ	Seizure-free
6		-	CBZ	No seizures 4 months after discontinuation of CBZ
7		-	CLB, CBZ	No seizures after addition of CBZ to intermittent CLB
8	6	-	LVT	Seizure-free
9		+	-	Seizure-free
10		-	CLB	Seizure-free
11		-	CLB	Seizure-free

EEG: Electroencephalography, HWE: Hot-water epilepsy, CBZ: Carbamazepine, CLB: Clobazam

Discussion

HWE is a benign form of reflex epilepsy with a relatively favorable response to treatment and prognosis. However, antiepileptic treatment may be required in the presence of spontaneous seizures and/or unresponsiveness to non-pharmacological measures.

According to the largest study from India with 279 cases, HWE was more common in children and among males than among females (male to female ratio of 2.6/1) (9). In our study, there was a male preponderance. In the literature, the age of onset of HWE varies extensively from infancy to adulthood. Satishchandra (9) reported the most frequent age of onset to be 1-5 years. In our study, the age at onset ranged from 1 to 12 years.

HWE is often benign in nature and the modification of bathing habits may suffice. In more severe cases with or without spontaneous seizures, pharmacological treatment is required. Moreover, spontaneous seizures may develop if left untreated, which is reported in 17-25% of patients (10). Satishchandra (9) reported the development of spontaneous seizures in 25.4% of cases within 1-3 years. The prevalence of spontaneous seizures may be as high as 35.3% (11). In our study, spontaneous seizures accompanied HWE in 36% of the cases.

The presence of febrile seizures, as well as a family history of epilepsy and febrile seizures, has been reported in cases of HWE (2,9,11). Satishchandra (9) reported febrile convulsions in 7% of their cases, a family history of epilepsy in 22.6%, and HWE in 7%. Meghana et al. (2) found febrile seizures in 14.2% of patients and a family history of epilepsy in 24.2% (HWE in 8%). In another study, 7.3% of patients had a family history of febrile seizures (11). In our study, three (37%) patients and two (18%) patients had a family history of HWE and febrile convulsions, respectively. Two patients with a family history of HWE were included in this study.

Concerning the semiology and EEG findings of HWE, Meghana et al. (2) reported focal-onset seizures with impaired awareness in all cases, with 58.5% showing focal-to-bilateral spreading. In another study, generalized tonic-clonic seizures were reported in 33% (8). We observed focal-onset seizures in 54% of patients, and focal-to-bilateral spreading was observed in 46% of our patients.

Radiological imaging of the brain is usually unremarkable for structural lesions (8). In a study of 38 cases, only three cases had incidental findings, such as a subarachnoid cyst, cavum septum pellucidum, or mega cisterna magna, in which the MRI of the brain was normal (11).

In the majority of cases with HWE, the interictal EEG is normal, with only 15-20% exhibiting diffuse abnormalities. In addition, spike wave discharges originating from the anterior temporal regions have been recorded in some

cases (3,8,9). EEG abnormalities were more frequently reported in those patients with accompanying spontaneous seizures than in those with HWE alone (11). In our study, EEG abnormalities were found in only two patients (18%) as focal changes in the temporal region, both of whom were free from spontaneous seizures.

Bathing with lukewarm water is the first-line treatment for HWE. Satishchandra (9) and Meghana et al. (2) reported that 38% and 13.3% of cases, respectively, remained seizure-free merely by the modification of their bathing habits. In the current study, 43% of our patients became seizure-free after the modification of their bathing habits. Generally, the frequency of HWE seizures is associated with the frequency of exposure to hot water over the head. However, in 5-10% of these patients, seizures may develop even during bathing, without hot water being poured over the head. Interestingly, self-induced seizures have been reported in approximately 10% of patients who experience intense pleasure from driving themselves until a loss of consciousness by compulsive exposure to hot water (8).

With the understanding that the pathogenesis of HWE is hyperthermic, similar to febrile seizures, intermittent prophylaxis has been proposed as an alternative treatment method along with bathing with lukewarm water (8). Satishchandra et al. (10) observed that prophylactic administration of clobazam 1.5 hours before bathing could be effective in HWE. In another report, 74.2% of patients remained seizure-free after intermittent clobazam treatment, and in 6.1% of the cases, seizures were reduced by more than 75% (12). Similarly, 75% of our patients benefited from clobazam with the resolution of seizures. Although intermittent clobazam prophylaxis before bathing may be effective in HWE, patients with spontaneous seizures require anti-seizure medications (12).

Study Limitations

The main limitation of the present study is its retrospective design. Seizure semiology was mainly based on patients' accounts. The small size of the patients from a single center main prevent generalizability of the results.

Conclusion

In conclusion, although HWE is a benign reflex epilepsy, HWE seizures should be identified and appropriately treated as there is always a risk of spontaneous seizures. At presentation, it is also important to determine the triggering factors and distinguish HWE from other epilepsies, so that the appropriate treatment of bathing with lukewarm water and intermittent clobazam prophylaxis can be initiated. Use of anti-seizure medications until seizure control is required in cases of spontaneous seizures.

Ethics

Ethics Committee Approval: This study was approved by the Institutional Review Board of Koç University (approval no.: 2023.077.IRB1.025, date: 09.03.2023).

Informed Consent: The study was waived from informed consent from patients due to its retrospective design.

Footnotes

Authorship Contributions

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

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

Financial Disclosure: The authors declared that this study received no financial support.

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J Pediatr Res 2024;11(4):225-31
DOI: 10.4274/jpr.galenos.2024.65625

Original Article

Frequency of Congenital Sucrase-Isomaltase Deficiency by Whole Exome Sequencing: Is It Really Rare?

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ABSTRACT

Aim: Congenital sucrase-isomaltase deficiency (CSID) is an autosomal recessive disease with a mutation in the sucrase-isomaltase (*SI*) gene and disaccharide maldigestion. With limited data, the estimated incidence of this disease is 57.59/10^6 births and its heterozygosity rate is 1/132. We aimed to evaluate cases who underwent whole exome sequencing (WES) analysis in the medical genetics unit with regards to the frequency and clinic of CSID.

Materials and Methods: The patients' files who underwent WES between 2018-2023 were evaluated retrospectively. The demographic characteristics, complaints, physical examination, and laboratory findings of those patients with *SI* gene mutations were recorded. Cases with mutations were recontacted and symptom questioning was performed.

Results: Mutations were detected in 25 (8.3%) of 300 patients who underwent WES analysis. One case had a compound heterozygous mutation, while 24 cases were heterozygous. The mean age was 22.4±17.6 years (1.8-52 years) and 16 (64%) were females. Nine of the cases (36%) were symptomatic.

Conclusion: Data on the frequency of CSID are insufficient and variable in the literature. The SI heterozygosity rate in our study was higher than some studies. There were similar rates when compared to the study conducted on symptomatic pediatric patients in our country. Although the data of our study includes a heterogeneous group to evaluate the frequency of CSID, it consists of a group with suspected genetic diseases rather than healthy individuals. Therefore, large-scale population-based studies are needed.

Keywords: Heterozygous carriers, prevalence, sucrase-isomaltase deficiency, sucrose, whole exome sequence analysis

Introduction

Congenital sucrase-isomaltase deficiency (CSID), first described in the 1960s, is a disorder of carbohydrate malabsorption caused by a homozygous or compound heterozygous mutation in the sucrase-isomaltase (*SI*) gene on chromosome 3q26 (1). Due to indigestible disaccharides (sucrose and isomaltose), symptoms such as osmotic foul-smelling diarrhea, abdominal pain, gas-bloating, and vomiting are frequently seen in the clinic presentation (2). Diagnostic difficulties of this disease and clinical

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Received: 11.06.2024 **Accepted:** 04.09.2024

resemblance to many chronic gastrointestinal system diseases delay its diagnosis. This may cause severe time and financial losses for the patients and their relatives.

Although duodenal biopsy enzyme activity is the gold standard for diagnosing CSID, it cannot be performed in many countries such as Turkey. Therefore, genetic analysis, which is relatively easily accessible, is used in clinically suspected patients for diagnosis (3).

There are few publications on the global prevalence of this disease. In a study by de Leusse et al. (4), CSID was the most common among congenital disaccharidase deficiencies, and its estimated prevalence was reported as 57.59 per 100,000 births. Regarding the prevalence between communities, serious differences are noteworthy, for example, the frequency of this disease in Ashkenazi Jews is 247/100,000, in Non-Finnish Europeans, it is 128/100,000, while in East Asia, it is 0.36/100,000 (5). It is thought that these differences are due to the lack of diagnosis of CSID cases in many countries due to diagnostic difficulties and low awareness and so they do not reflect the true prevalence rates (6).

In recent years, the genetic and clinical structure of this disease has come to the fore again, with studies showing that cases with heterozygous SI mutations may also be symptomatic (7). de Leusse et al. (4) stated that the estimated prevalence of CSID heterozygosity was 1/132. In a recent study in Turkey, the rate of CSID in pediatric patients was 11%, while other studies were in the form of case reports (8-10).

With developments in genetics, whole exome sequence (WES) analysis can be performed in most genetics laboratories. In this way, many rare diseases which were difficult to diagnose can now be diagnosed more easily (11). At the same time, many pathogenic variants can be detected incidentally in addition to the clinically predicted diseases.

This study aimed to contribute to the literature by retrospectively evaluating the frequency and clinic presentations of *SI* gene mutations in those patients who underwent WES for any indication in the medical genetics unit of our hospital.

Materials and Methods

The results of those cases who underwent WES in our hospital for any reason between January, 2018 and October, 2023 were retrospectively analyzed. The demographic characteristics of those patients with SI mutations, reasons for performing WES, complaints, physical examination, and laboratory findings were obtained from the hospital files. Cases with mutations were contacted and symptom questioning was performed again regarding CSID.

In order to obtain genomic DNA, 2 cc peripheral venous blood samples were taken from the patients who underwent WES analysis into tubes with EDTA. From the blood taken, DNA isolation was performed manually with the application procedure of a peripheral blood lymphocyte cells DNA isolation kit (Roche) in the medical genetics laboratory. The extracted DNA was maintained at -20 degrees until it was used. After adaptor ligation was performed on the ends of the DNA sequences to recognize and separate the DNA samples obtained from peripheral blood planned to be analyzed by the next generation sequence (NGS) and WES analysis, a purification process was applied. The protocol specified in the kit instruction manual was applied for the DNA indexing stage attached to the purified adapter. The pool (library) created for the indexed sample DNAs was combined in the tube at the appropriate concentration, loaded into the device cartridge, and taken to the sequencing process with the Illumina Next Generation Sequencing device. The Illumina NextSeq platform was used for NGS. In this study, the raw data obtained by working with Twist Bioscience NGS diagnostic kits on the Illumina NextSeq platform were analyzed according to the reference genome GRCh37(h19) in a web-based bioinformatics program (https://seq.genomize.com/V.6.2.3). Clinvar, VarSome and Franklin databases were used in the evaluation of variations for pathogenicity classification, taking into account the recommendations of American College of Medical Genetics and Genomics (ACMG) Standards and Guidelines, 2015. Variant region reads were evaluated using the Integrative Genomics Viewer program. With regards to detected variants, the ESP6500 was checked with the ExAC and GnomAD Exome study frequency values. Variants in the *SI* gene, classified as pathogenic, likely pathogenic, and variant of uncertain significance (VUS), were evaluated.

This study was conducted according to the Principles of the Declaration of Helsinki. Ethical approval for this study was obtained from the Süleyman Demirel University Local Ethics Committee (decision date: 28.09.2022, approval no.: 262). Informed consent was obtained from all participants.

Statistical Analysis

IBM SPSS (Statistical Package for the Social Sciences) version 23.0 (IBM Corp.; Armonk, NY, USA) program was used to evaluate the data in our study. For descriptive statistics, parametric tests assumptions are provided for numerical variables, standard deviations, or medians

(minimum-maximum). Categorical variables are given as numbers (n) and percentages (%).

Results

During our study period, WES was performed on 300 patients in the medical genetics unit at our center. The *SI* gene mutation was detected in 31 patients. These mutations were evaluated with the ACMG, ClinVar and Franklin Genoox databases, and those patients with benign or likely benign variants in at least one database (n=6) were excluded. A total of 25 patients, 24 heterozygous and one compound heterozygous, were included in this study (Figure 1).

While pathogenic/likely pathogenic variants were observed in 5 cases, the majority of cases (n=20, 80%) were VUS variants. Some variants were present in more than one patient. The 'c.1919A>G (p.Glu640Gly)' variant was seen in three cases, and the 'c.1730T>G (p.Val577Gly)', 'c.2864G>A (p.Cys955Tyr)' and 'c.2923T>C (p.Tyr975His)' variants were detected in two cases each (Table I).

Twenty-one different SI variants were detected in 25 cases. It was observed that 6 variants found in 6 cases were intronic, 15 variants found in 20 cases were exonic, and one case had both intronic and exonic variants.

When the WES indications of the cases with mutations were evaluated, while neurological reasons (n=9, 36%) were the primary reasons, other reasons included cancer screening (n=5, 20%), syndromic appearance (n=4, 16%), immunodeficiency (n=3, 12%), hematological (n=2, 8%) and musculoskeletal (n=2, 8%) diseases.

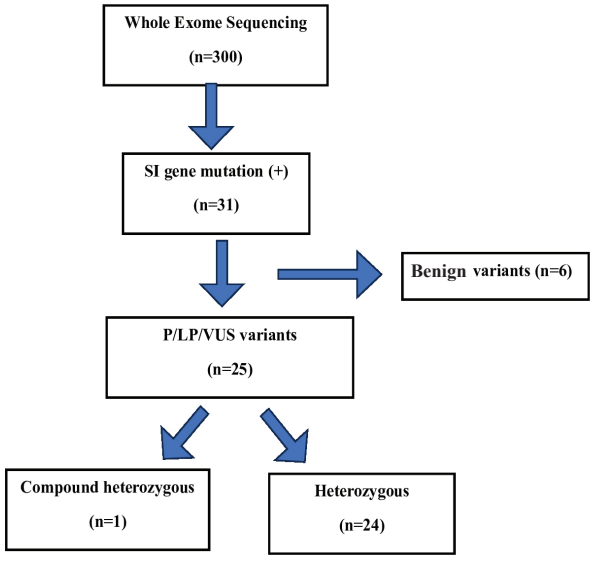

Figure 1. Algorithm of patients included in the study

The mean age was 22.4±17.6 years (1.8-52 years) and 16 (64%) were females. CSID-related symptoms were present in a total of 9 cases (36%), one with a compound heterozygote and 8 with a heterozygous mutation. Sixteen cases (64%) were in the pediatric age group and constituted the majority (7/9) of symptomatic cases.

There was only one patient with a compound heterozygous mutation and symptoms. This case was a 12-year-old male patient with *SI* gene 'c.3218G>A,p.Gly1073Asp/c.1147-177A>G' compound heterozygous mutation. The 'c.3218G>A,p.Gly1073Asp' variant was classified as "pathogenic" by the ClinVar mutation database and "likely pathogenic" by Franklin Genoox. The other 'c.1147-177A>G' variant was classified as "VUS" by the ACMG and Franklin Genoox databases, and "not available" by ClinVar. When questioned regarding CSID-related symptoms, there was intermittent abdominal pain with no nutritional relationship clearly stated.

When the symptomatic heterozygous cases (n=8) were evaluated, two adult cases had abdominal pain and dyspepsia. In the pediatric cases, growth retardation (n=3), diarrhea (n=2) and abdominal pain (n=1) were detected (Table II).

The adult patients were re-evaluated and it was stated that their symptoms were not significant and did not reduce the quality of their daily life. When the symptoms were questioned for the pediatric patients, it was observed that there were no constant complaints.

Discussion

CSID is considered an autosomal recessive inherited and rare disorder. However, more than 40 mutations have been identified recently, and sucrase-isomaltase enzyme deficiency is the most common disaccharidase deficiency (12). Due to difficulties in diagnostic testing and changes in symptoms with age, the true prevalence of this disease is likely to be underestimated and may be more common than expected (13).

The clinical presentation often occurs as severe watery diarrhea, abdominal distention, bloating, inability to gain weight, irritability, and diaper dermatitis due to exposure to sucrose or starch after complementary feeding in infancy (14). Significant symptoms in the first years of life may be intermittent diarrhea, abdominal pain, and gas-bloating, with sweet and fruit avoidance in later years. Clinical findings such as diarrhea-predominant irritable bowel syndrome can be seen in adolescents and adults (15).

More pronounced clinical symptoms in children have been associated with a shorter transit time in the small intestine and a lower absorption capacity of the colon (16). In our study, the majority of our symptomatic cases (n=7, 77%) were in the pediatric age group. Other factors affecting the symptoms include homozygous or heterozygous *SI* gene mutation, residual enzyme activity, and the amount of sugar and starch consumed (17).

Although symptoms have been reported in cases with homozygous or compound heterozygous mutations due to the inheritance pattern of the disease, there have been reports in recent years that heterozygous individuals may also be symptomatic (18). In cases with heterozygous mutations, dyspeptic complaints such as abdominal pain and nausea-vomiting can be seen rather than osmotic diarrhea, and it can be diagnosed as a functional gastrointestinal disease (19). In our study, the case with the compound heterozygous mutation was symptomatic, as expected. In addition, 8 of the 27 heterozygous cases were symptomatic. Complaints of abdominal pain and dyspepsia were observed in the adults, similar to the literature. In the pediatric cases with heterozygous mutation, the symptoms were variable. This variability can be explained by the WES indications of the patients and their primary underlying diseases. While growth retardation may be observed in cases with a syndromic appearance, diarrhea may also be present in patients with immunodeficiency. It does not seem possible to directly relate these symptoms to CSID.

Table I. Characteristics of cases with SI mutation

Case	Gender/Age	SI Variant 1/SI Variant 2	Exon/Intron	ACMG	ClinVar	Franklin Genoox	Symptom
1	M/12	**c.3218G>A/ c.1147-177A>G**	(p.Gly1073Asp)/ intronic	VUS VUS	P NA	LP VUS	+
2	M/8	**c.1730T>G**	(p.Val577Gly)	VUS	P	P	-
3	F/44	**c.1730T>G**	(p.Val577Gly)	VUS	P	P	+
4	M/16	**c.4099A>G**	(p.Arg1367Gly)	VUS	LP	LP	-
5	M/6	**c.1544G>T**	(p.Gly515Val)	VUS	NA	LP	+
6	M/11	**c.1919A>G**	(p.Glu640Gly)	VUS	VUS	VUS	-
7	M/45	**c.1919A>G**	(p.Glu640Gly)	VUS	VUS	VUS	+
8	M/46	**c.1919A>G**	(p.Glu640Gly)	VUS	VUS	VUS	-
9	F/15	**c.1-15C>A**	Intronic	VUS	VUS	VUS	-
10	F/3.5	**c.2923T>C**	(p.Tyr975His)	VUS	VUS	LB	+
11	M/16	**c.2923T>C**	(p.Tyr975His)	VUS	VUS	LB	+
12	F/28	**c.4951G>A**	(p.Val1651Ile)	VUS	VUS	LB	-
13	F/1.5	**c.170C>T**	(p.Pro57Leu)	VUS	NA	VUS	+
14	F/16	**c.1888-160_1888 159delinsGC**	Intronic	VUS	NA	VUS	-
15	M/14	**c.2864G>A**	(p.Cys955Tyr)	VUS	NA	VUS	+
16	F/6	**c.2864G>A**	(p.Ile1191Leu)	VUS	NA	VUS	-
17	F/3.5	**c.3571A>C**	(p.Cys955Tyr)	VUS	NA	VUS	-
18	F/8	**c.3562G>A**	(p.Val1188Ile)	VUS	NA	VUS	-
19	F/8	**c.3099+73A>T**	Intronic	VUS	NA	VUS	+
20	F/45	**c.374-4T>C**	Intronic	VUS	NA	VUS	-
21	F/17	**c.3149T>A**	(p.Ile1050Asn)	VUS	NA	VUS	-
22	F/45	**c.5422C>T**	(p.Arg1808Cys)	VUS	NA	VUS	-
23	F/47	**c.4457C>T**	(p.Thr1486Met)	VUS	NA	VUS	-
24	F/47	**c.5111G>A**	(p.Arg1704Gln)	VUS	NA	VUS	-
25	F/52	**c.2737-33dup**	Intronic	VUS	NA	VUS	-

SI: Sucrase-isomaltase, VUS: Variant of uncertain significance, NA: Not available, ACMG: American College of Medical Genetics and Genomics, LB: Likely benign, LP: Likely pathogenic

Table II. Characteristics of symptomatic cases

Case	Gender/Age	WES indication	Mutation	Symptom
1	M/12	Musculoskeletal	P/VUS-compound heterozygous	Abdominal pain
2	M/6	Immunodeficiency	LP- heterozygous	Diarrhea
3	M/14	Immunodeficiency	VUS- heterozygous	Diarrhea
4	F/1.5	Syndromic	VUS- heterozygous	Growth retardation
5	F/3.5	Syndromic	VUS- heterozygous	Growth retardation
6	F/8	Musculoskeletal	VUS- heterozygous	Growth retardation
7	M/16	Neurological	VUS- heterozygous	Abdominal pain
8	F/44	Neurological	P- heterozygous	Abdominal pain
9	M/45	Neurological	VUS- heterozygous	Dyspepsia

P: Pathogenic, LP: Likely pathogenic, VUS: Variant of unknown significance, WES: Whole exome sequencing

It has been stated in the literature that symptoms are not only related to *SI* gene mutation, but that clinical presentation may vary due to multifactorial reasons (2). Similarly, in our study, heterogeneity was observed in the symptom distribution of cases with the same mutation. Although it is known that symptoms are often evident at younger ages, the symptomatic cases of the 'c.1919A>G (p.Glu640Gly)' and 'c.1730T>G (p.Val577Gly) variants were adults, while the pediatric cases with the same mutation were asymptomatic. It has shown that residual enzyme activity may lead to variability and different clinical conditions in individuals with the same mutation. For this reason, in cases where enzyme activity cannot be measured, it becomes difficult to diagnose and treat CSID.

The gold standard method for the definitive diagnosis of this disease is the measurement of enzyme activity from duodenal mucosa samples. Other noninvasive tests which support the diagnosis include the sucrose challenge test or the ^{13}C-sucrose breath test (20). However, neither enzyme activity nor ^{13}C-sucrose breath tests can be performed in Turkey. In the study by Karakoyun et al. (10), five cases were diagnosed with a sucrose challenge test after a detailed nutritional history and the relationship between symptoms was determined. Another diagnostic method, *SI* gene mutation analysis, is not primarily preferred due to its high cost, but it can be used when other diagnostic methods are not available (21). Taskin et al. (8) also used next-generation sequencing (NGS) analysis for the diagnosis of CSID, referencing the recommendations of the Alaska Native Medical Center guidelines.

The use of NGS methods such as WES has become increasingly common in the detection of possible genetic diseases in cases with diagnostic difficulties. While this method is frequently used for neurological findings (35%), and multiple congenital anomalies (24%), it can also be preferred for immunodeficiency, musculoskeletal system anomalies, growth retardation, hematopoietic system, and hearing disorders (22). In our study, similar to the literature, neurological causes were among the most common WES indications. During the analysis, genetic variants related to other diseases can sometimes be detected incidentally. As a result of the WES analysis of 3,040 cases by Retterer et al. (22), secondary pathogenic variants were reported in 6.2% (n=129) of the cases (23). We detected the secondary pathogen SI variant rate as being 1.6% (5/300), but we did not evaluate this rate for other diseases. In addition to pathogenic variants, we also evaluated cases with SI mutation regarding exon and intronic variants. Examining intronic variants as well as exonic variants is performed due to the increasing number of publications in recent years indicating that intronic variants may be pathogenic (24). We observed that 2 of 6 cases (30%) with the intronic variant were symptomatic.

In our study, mutations in the *SI* gene were detected in 8.3% of those patients who underwent WES; 0.33% were compound heterozygous, while 8% had heterozygous SI variants. In a study evaluating the estimated prevalence of congenital disaccharidase deficiencies, the prevalence of heterozygous CSID was reported as being 1/132, and our results were found to be quite high compared to the limited

studies in the literature (4). The drawback of our study was that our study group may not have reflected the population in terms of specifying the frequency of CSID, since it included individuals with more genetic diseases.

Taskin et al. (8) reported the frequency of CSID as being 11.7% (1 homozygous, 10 heterozygous mutations) via the NGS method in 94 patients with chronic nonspecific diarrhea. This rate was higher than our results, which can be explained by the fact that genetic analysis was performed on symptomatic pediatric cases. We consider that the heterogeneous distribution of our study may be more consistent in reflecting the frequency of CSID.

Conclusion

In conclusion, the variable clinical presentations in heterozygotes, in contrast to the OR inheritance of CSID, may require a redefinition of the genetic pattern of this disease. However, there is still insufficient data on the prevalence of CSID. There are widely varying results in the literature. Although the data of our study includes a heterogeneous group, larger-scale population-based studies are needed in order to clarify the enzymatic activity and genotype-phenotype relationship.

Ethics

Ethics Committee Approval: This study was conducted according to the Principles of the Declaration of Helsinki. Ethical approval for this study was obtained from the Süleyman Demirel University Local Ethics Committee (decision date: 28.09.2022, approval no.: 262).

Informed Consent: Informed consent was obtained from all participants.

Footnotes

Authorship Contributions

Surgical and Medical Practices: F.İ.I., Concept: F.İ.I., M.A., Design: F.İ.I., M.A., Data Collection or Processing: F.İ.I., H.Ö., H.S., Analysis or Interpretation: H.Ö., Literature Search: F.İ.I., M.A., Writing: F.İ.I.

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

Financial Disclosure: The authors declared that this study received no financial support.

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J Pediatr Res 2024;11(4):232-40
DOI: 10.4274/jpr.galenos.2024.98048

Original Article

The Reliability and Validity Study of The Partner Breastfeeding Influence Scale

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ABSTRACT

Aim: This study aimed to assess the validity and reliability of the Partner Breastfeeding Influence Scale (PBIS) in the Turkish population.

Materials and Methods: This research was carried out as a methodological descriptive cross-sectional study. The study population consisted of 301 fathers who attended a new-born outpatient clinic at a children's hospital from July, 2018 to December, 2018. The data for this study were gathered via the Person Identification Form and the PBIS. The scale comprises five subscales and a total of 37 items. The subscales include Breastfeeding Savvy, Helping, Appreciation, Breastfeeding Presence and Responsiveness. The validity of this scale was assessed by exploratory and confirmatory factor analyses.

Results: The Cronbach's alpha correlation coefficient was determined to be 0.95 for the entire scale and between 0.75 to 0.83 for the subscales. The exploratory factor analysis accounted for 59.09% of the overall variation. The factor load values of the scale, as determined using confirmatory factor analysis, ranged from 0.28 to 0.82. Goodness of Fit Index, Normed Fit Index, Non-Normed Fit Index, and Comparative Fit Index were greater than 0.90, whereas root mean square error of approximation was less than or equal to 0.08.

Conclusion: PBIS is a credible and dependable instrument applicable in Turkish culture.

Keywords: Breast milk, breastfeeding, father, reliability, validity

Introduction

Breast milk is a natural source of nutrition which provides all the fluids, energy, and nutrients necessary for a baby's physical, mental, and intellectual development. It is highly bioavailable, easy to digest, and helps reduce morbidity and mortality in new-borns (1-5). However, social and economic developments have changed the roles of individuals within the family (6). The roles of the mother, father, and child, as family members, have evolved with regards to societal norms and over time (7). A rising number of professional women re-enter the workforce post-childbirth, and the heightened focus on gender equality has elevated the fathers' involvement in their children's lives (6). Consequently, breastfeeding is not exclusively a matter between the mother and infant; fathers also play an essential role in assisting the mother and facilitating the initiation and maintenance of breastfeeding (8).

The current literature emphasizes the importance of consulting and educating mothers about breastfeeding to facilitate a healthy breastfeeding process. Including fathers in this process makes mothers more determined to start and sustain breastfeeding (7-10). Breastfeeding rates are higher

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Received: 17.05.2024 **Accepted:** 04.09.2024

when partners and families provide support. Moreover, mothers experience fewer challenges and are also better equipped to cope with them when support is present (8,11,12). A comfortable and peaceful environment, supported by the father, helps the mother feel emotionally at ease, which is vital for successful breastfeeding (13,14). Furthermore, the father can enhance the mother's motivation to maintain breastfeeding (12,15,16).

If fathers are included in breastfeeding education, solutions to breastfeeding challenges can be more effectively addressed, thus promoting exclusive breastfeeding and continuation beyond six months. This can play a critical role in increasing breastfeeding rates (9,17,18).

Despite the recognized importance of the father's influence on breastfeeding, studies evaluating this influence are limited in the literature. There is currently no scale in our country for assessing the father's influence on breastfeeding. The Partner Breastfeeding Influence Scale (PBIS) has the potential to raise awareness among fathers regarding breastfeeding. Consequently, fathers may offer greater assistance to mothers, resulting in higher breastfeeding rates.

Materials and Methods

Setting and Participant

This was a cross-sectional study designed to evaluate the validation and reliability of the Turkish adaptation of the PBIS. This study received approval from the Non-Interventional Studies Ethics Committee of Manisa Celal Bayar University (approval no.: 20.478.486, date: 23.05.2018). All procedures conducted in studies involving human subjects adhere to the ethical criteria set forth by the institutional and/or national research committee, as well as the 1964 Helsinki Declaration and its subsequent revisions or equivalent ethical guidelines.

The study population consisted of fathers who attended a new-born polyclinic from July, 2018 to December, 2018 for their infants. Among these, 301 fathers who fulfilled the inclusion criteria, completed the scale and consented to participate formed the study sample.

In validity and reliability studies, it is advised that the sample size be five to ten times the number of items in the scale (19-21). According to this recommendation, the sample size was determined to be five to ten times the total number of items in the scale utilized in our study. The fathers were apprised of the study's objectives and requirements. Informed written consent was acquired from each father who consented to participate after reviewing the consent form. During the examination of their infant, the fathers who consented to participate filled out the study forms.

Data Collection Tools

The "Individual Identification Form" and the "Partner Breastfeeding Influence Scale" served as instruments for data collection.

Individual Identification Form

This form was developed by the researchers in accordance with the literature. It contains 13 questions, including the father's age, educational status, occupation, number of children, the age and sex of those children currently breastfeeding, and other related questions (Table I).

PBIS

This scale was initially created by Rempel and Rempel (22). It includes 37 items and evaluates how often fathers engage in specific behaviours while their partners are breastfeeding. The scale employs a 5-point Likert-type format: 1 represents "Not at all", 2= "Rarely", 3= "Sometimes", 4= "Often" and 5= "Very often". The scale has five subscales: Breastfeeding Savvy, Helping, Appreciation, Breastfeeding Presence, and Responsiveness.

- Breastfeeding Savvy includes items related to learning and discussing breastfeeding knowledge.
- Helping includes items related to direct and indirect support, such as assisting with household chores, childcare, and partner care during the breastfeeding period.
- Appreciation includes items related to encouraging the mother in breastfeeding and expressing gratitude to her.
- Breastfeeding presence encompasses elements pertaining to the father's supportive involvement during breastfeeding.
- Responsiveness encompasses aspects pertaining to the father's regard for the mother's choices and his attunement to her requirements.

Higher scores on the overall scale indicate a greater influence of the father on breastfeeding. The maximum score on this scale is 185, while the minimum is 37.

Data Collection

Language Validity

Two colleagues and an English/Turkish translator worked on the Turkish translation of the scale. The Turkish

version was then retranslated into English by two linguists. The original and retranslated scales were reviewed by both the linguists and researchers, who reached a consensus on each item in order to finalize the scale.

Content Validity

Expert evaluations were solicited in order to determine the content validity of the scale. The Turkish translation of the scale was submitted to ten experts in their respective domains for evaluation. Following deliberation among the experts, numerous terms were amended, resulting in the final iteration of the scale. The Content Validity Index (CVI) was employed to assess the experts' evaluations regarding content validity (23). Experts evaluated each item on a scale from 1 to 4, where 1 indicated "Not suitable", 2 indicated "Mildly suitable", 3 indicated "Suitable but needs some changes", and 4 indicated "Very suitable".

Construct Validity

Exploratory and confirmatory factor analyses (CFAs) were performed in order to evaluate the construct validity of the scale. The omitted items were those which did not load well onto any of the subscales or those which did not conceptually fit well with the identified subscales.

Determination of Reliability

Cronbach's Alpha reliability coefficient and item-total score analyses were conducted in order to assess the scale's reliability.

Statistical Analysis

Statistical analysis was conducted using SPSS v25.0 (24), with p-values less than 0.05 deemed significant. The Cronbach's alpha coefficient was calculated in order to assess the internal consistency of the scale. A correlation analysis of item-total scores was performed in order to assess the impact of each item on the overall score. Descriptive factor analysis was employed to assess the item-factor relationship. Ultimately, CFA was conducted to ascertain whether the items and subscales accurately represented the scale's structure.

Results

Study Population

The participants' socio-demographic data are presented in Table I. The average age of the fathers was 31.69±5.60 years. Of the fathers, 42.5% had a bachelor's degree, 49.8% worked in the private sector, and 90.7% of the families resided in an urban area as opposed to a rural one (Table I).

Table I. Distribution of sociodemographic data on fathers (n=301)

Socio-demographic data on fathers	n (number)	% (percentage)
Mean age	31.69±5.60 (min.: 20, max.: 55)	
Educational level		
Primary	14	4.7
Secondary	26	8.6
High school	114	37.9
Bachelor	128	42.5
Graduate	19	6.3
Work status		
Yes	301	100
No	-	-
Residence of family		
City	273	90.7
Countryside	28	9.3
Occupation		
Public official	86	28.6
Private sector	150	49.8
Freelance	65	21.6
Income status		
Negative profit-loss	64	21.3
Balanced profit-loss	178	59.1
Positive profit-loss	59	19.6
Working period		
Day time	265	88
Night time	1	0.3
Both	35	11.6
Work status of mother		
Yes	165	54.8
No	136	45.2
Occupation of mother		
Public official	85	28.2
Private sector	74	24.6
Freelance	6	2
None	136	45.2
Number of children		
1	132	43.9
2	127	42.2
3	32	10.6
4	9	3
5	1	0.3
Rank of child taking breast milk		
1st	147	48.8
2nd	114	37.9
3rd	31	10.3
4th	8	2.7
5th	1	0.3
Age of child taking breast milk		
<1 month	84	27.9
1 up to 6 months	128	42.5
6 up to 12 months	63	20.9
12 up to 18 months	15	5
18 up to 24 months	10	3.3
24 months or above	1	0.3
Gender		
Female	159	52.8
Male	142	47.2
Total	301	100.0

Validity Analysis

Language Validity

The initial phase of this study involved the evaluation of language validity. The scale was initially translated from English to Turkish by a translator skilled in both languages. The translated version was then evaluated by five experts who were also fluent in both languages. After the evaluations, the researcher and experts collaborated to create a common text. This text was subsequently retranslated into English by an impartial individual.

Content Validity

CVI was assessed utilizing the Davis method. Ten experts in Pediatric Nursing and Women's Health and Diseases Nursing assessed the content validity of each item on the Turkish version of the scale, using a rating scale from 1 to 4 points. This expert group should comprise a minimum of 3 and a maximum of 20 individuals. Utilizing the Davis technique, the specialists evaluated each item as: a) appropriate, b) needs revision, or c) requires serious revision. The CVI for each item was determined by dividing the number of experts who chose options (a) or (b) by the total number of experts (25). The item-level-CVI (I-CVI) for the scale items was assessed at 0.92, employing a four-point grading system to identify inappropriate items. The scale-level-CVI (S-CVI) was determined to be 0.98, signifying high content validity at the scale level.

Construct Validity

Exploratory Factor Analysis

Kaiser-Meyer-Olkin (KMO) and Bartlett's tests were performed to evaluate the data's homogeneity and appropriateness for factor analysis. These findings demonstrated that the data were homogeneous and the variances were suitable for factor analysis. Prior to conducting the exploratory factor analysis of the PBIS, Bartlett's test yielded χ^2=5688.606, the KMO statistic was 0.94, and $p<0.001$. The data were classified into five subscales. The breastfeeding savvy subscale contributed 40.08% to the total variance, the helping subscale contributed 7.25%, the appreciation subscale contributed 4.67%, the breastfeeding presence subscale contributed 3.65%, and the responsiveness subscale contributed 3.42%.

CFA revealed that the factor loadings for the breastfeeding savvy subscale ranged from 0.50 to 0.65, for the helping subscale, it was from 0.54 to 0.69, for the appreciation subscale, it was from 0.48 to 0.70, for the breastfeeding presence subscale, it was from 0.56 to 0.68, and for the responsiveness subscale, the range was from 0.40 to 0.66 (Table II).

CFA

The CFA revealed that factor loadings in the breastfeeding savvy subscale varied from 0.28 to 0.60, in the helping subscale, it was from 0.37 to 0.59, in the appreciation subscale, it was from 0.42 to 0.82, in the

Table II. Factor loadings for the five factors

The partner breastfeeding influence scale	Item number	Factors	Factor loadings	Exploratory factor analysis (%)
Discuss or negotiate with your partner about how long to continue breastfeeding	Item 1	Factor 1	0.554	40.08
Discuss with your partner ideas for trying to solve breastfeeding problems or making suggestions for creative or different ways to make breastfeeding work better	Item 3		0.612	
Learn more about breastfeeding by reading books or articles on breastfeeding.	Item 10		0.647	
Tell your partner your opinion about how long you think that she should breastfeed.	Item 11		0.629	
Speak up in support of your partner or defend breastfeeding when someone makes a negative breastfeeding comments.	Item 14		0.654	
Help your partner get assistance from others for solving breastfeeding problems or improving breastfeeding (for example, by asking others for advice, getting professional help, or going along to get help)	Item 15		0.556	
Remind your partner of the benefits that breastfeeding has for her or for your baby (for example, it saves money, it is easier than bottle feeding)	Item 23		0.580	
Show patience and a willingness to wait for your opportunity to feed the baby.	Item 30		0.526	
Support your partner's attendance at a breastfeeding support group	Item 31		0.502	

Table II. Continued

The partner breastfeeding influence scale	Item number	Factors	Factor loadings	Exploratory factor analysis (%)
Help out with or take care of other childcare tasks with the baby (for example, rocking, soothing, responding to the baby's cries, changing diapers)	Item 4	Factor 2	0.639	7.25
Give something up in order to make breastfeeding easier (for example, be willing to set aside hobbies or preferred activities, take time off work, stop on a car trip)	Item 7		0.566	
Help out with other household tasks and responsibilities to free up your partner's time and energy.	Item 9		0.541	
Help out with breastfeeding at night (for example, bring the baby, put the baby back to bed)	Item 16		0.607	
Care for your baby during and after breastfeeding is done (for example, burp the baby, change the diaper)	Item 17		0.694	
Try to improve your partner's health and nutrition (for example, cooking nutritious meals, helping to avoid foods as agreed)	Item 28		0.571	
Give your partner a break from the baby (for example, encourage personal time away, take care of the baby so that she can have time to herself)	Item 29		0.593	
Encourage your partner to do her best when it comes to breastfeeding and let her know that she is not less of a mother if she feels like quitting	Item 12	Factor 3	0.623	4.67
Praise your partner for breastfeeding and let her know that what she is doing is a beautiful, worthwhile thing	Item 18		0.650	
Let your partner know that breastfeeding is natural and/or give her the message that she is breastfeeding because (that is who she is) she wants the best for her baby	Item 19		0.699	
Listen to and encourage your partner when she is feeling frustrated or discouraged about breastfeeding	Item 22		0.605	
Show appreciation that your partner is breastfeeding (for example, bring her flowers, take her out for dinner)	Item 32		0.515	
Tell your partner that you value and support her mothering decisions and intuitions around breastfeeding	Item 36		0.478	
Try to improve the breastfeeding experience by getting equipment or supplies ready for breastfeeding (for example, preparing a breastfeeding pump, get things such as a pillow that will make your partner comfortable)	Item 5	Factor 4	0.643	3.65
Act attentively towards your partner during breastfeeding (for example, bring your partner food or drink, a book, or massage your partner's shoulders or back)	Item 6		0.651	
Quietly share time and watch or hold your partner during breastfeeding	Item 13		0.626	
Physically help with breastfeeding related activities (for example, check the baby's latch or position, breast massage, hold a breast pump, help with breastfeeding aids)	Item 20		0.586	
Help create a quiet, pleasant environment for breastfeeding	Item 21		0.561	
Show pleasure and satisfaction while your partner is breastfeeding (for example, watch, smile)	Item 24		0.680	
Make it easy for your partner to breastfeed while entertaining company or visiting others (for example, by entertaining company while your partner breastfeeds or by joining your partner in a private place at a social event)	Item 2	Factor 5	0.578	3.42
Respond sensitively and positively to sexual issues (for example, understand your partner's feelings about not having sexual relations more than she wants, understand her feelings about touching her breasts, be flexible in sleeping arrangements and allow the baby to sleep in your bed)	Item 8		0.486	
Be patient and understanding of the time it takes to breastfeed and don't get upset if the other housework is not done	Item 25		0.402	
Show your comfort with breastfeeding in public (for example, malls, restaurants) and help her feel comfortable too	Item 26		0.663	
Pay attention to how and how much your partner wants you to participate in breastfeeding	Item 27		0.580	
Total scale				59.09

breastfeeding presence subscale, it was from 0.46 to 0.61, and in the responsiveness subscale, the range was from 0.28 to 0.55 (Table III). The model fit indicators for the PBIS were as follows: root mean square error of approximation (RMSEA)=0.08, Goodness of Fit Index (GFI)=0.94, Comparative Fit Index (CFI)=1.00, Incremental Fit Index (IFI)=1.06, Normed Fit Index (NFI)=1.00, Non-Normed Fit Index (NNFI)=1.06, χ^2=1448.52, and degree of freedom=485 (p<0.001). The reference values for fit indices, as outlined by Schermelleh-Engel et al. (26), are given in Table III.

Reliability Analysis

In order to assess internal consistency as a reliability indicator of the PBIS and its subscales, the Cronbach's alpha coefficient was calculated. The subscale coefficients ranged between 0.75 and 0.95, and the overall scale coefficient was 0.95, indicating high reliability. All subscales exhibited Cronbach's alpha values exceeding 0.70 (Table IV). The item-total correlation coefficient varied from 0.240 to 0.721. The correlation coefficients for the breastfeeding savvy subscale ranged from 0.525 to 0.741, for the helping subscale, it was from 0.608 to 0.764, for the appreciation subscale, it was from 0.610 to 0.855, for the breastfeeding presence subscale, it was from 0.634 to 0.778, and for the responsiveness subscale, the range was from 0.642 to 0.731. All values were determined to be statistically significant (p<0.001).

Table III. Model fit indices of confirmatory factor analysis

Models	Acceptable fit	Value found in the analysis Model 5 (five sub-dimensional model)
X^2/df	2<X^2/df<3	2.98 (Acceptable)
RMSEA	0.05<RMSEA<0.10	0.08 (Acceptable)
GFI	0.90<GFI<0.95	0.94 (Acceptable)
CFI	0.95<CFI<1.00	1.00 (Acceptable)
IFI	0.90<IFI<0.95	1.06
NFI	0.90<NFI<1.00	1.00
NNFI	0.90<NNFI<0.95	1.06
RFI	0.90<RFI<1.00	0.92 (Acceptable)

RMSEA: Root mean square error of approximation, GFI: Goodness of Fit Index, CFI: Comparative Fit Index, IFI: Incremental Fit Index, NFI: Normed Fit Index, NNFI: Non-Normed Fit Index, RFI: Relative Fit Index, df:degree of freedom

Discussion

This study analysed the validity and reliability of the Turkish version of the PBIS scale, created by Rempel and Rempel (22). Factor analysis was performed in order to assess the scale's validity, internal consistency was evaluated for construct validity, and test-retest reliability coefficients were computed.

Expert opinions were solicited from ten individuals to assess language and content validity. Both item-level and scale-level fit indices exceeded 0.90. The CVI was employed to assess the expert opinions. The item-level CVI varied from 0.9 to 1.0, while the scale-level CVI was 0.98 (Kendall's W value=0.163; p<0.05). The literature indicates that a CVI exceeding 0.80 is preferable for both item-level and scale-level assessments (23). The findings indicate that the PBIS, encompassing all 37 original items, is appropriate for Turkish culture and demonstrates content validity.

The compatibility of data and sample size for factor analysis were assessed utilizing the KMO coefficient and Bartlett's test. Prior to conducting descriptive factor analysis, Bartlett's test yielded a result of χ^2=5,688.606 for the PBIS, and the KMO coefficient was 0.94 (p<0.001). This outcome signified that the sample size was adequate for factor analysis and that the data distribution was uniform. The analysis identified a five-factor structure

Table IV. Cronbach's alpha reliability coefficient of the partner breastfeeding influence scale and subscales (n=301)

Scale and subscales	Number of items	Min.-Max. points	X±SD	Cronbach's α reliability coefficient
The partner breastfeeding influence scale	33 items	33-165	114.5±21.05	0.95
Breastfeeding savvy (items 1, 3, 10, 11, 14, 15, 23, 30, 31)	9 items	9-45	29.5±6.1	0.82
Helping (items 4, 7, 9, 16, 17, 28, 29)	7 items	7-35	24.3±5.09	0.83
Appreciation (items 12, 18, 19, 22, 32, 36)	6 items	6-30	21.3±4.3	0.83
Breastfeeding presence (items 5, 6, 13, 20, 21, 24)	6 items	6-30	20.8±4.5	0.83
Responsiveness (items 2, 8, 25, 26, 27)	5 items	5-25	18.4±3.4	0.75
Omitted items (items 33, 34, 35, 37)				

X: Mean, SD: Standard deviation, Min.-Max.: Minimum-Maximum

with an eigenvalue exceeding 1.00, accounting for 59.9% of the total variance. This signified that the PBIS attained a satisfactory degree of total variance in this study. Upon analysing the primary components of the subscales, factor load values were determined to be at medium or high levels. This analysis substantiated the construct validity of the PBIS.

After the validity factor analysis of this study, the RMSEA was found to be 0.08. The GFI, NNFI, NFI, and CFI of the factor loads of the subscales were higher than 0.90 (Figure 1). These values demonstrate that the database aligns with the model and validate the five-factor structure. The items and subscales exhibit correlation with the overall scale, and each item within the subscales adequately delineates its respective factor. These results support the structural validity of PBIS and suggest that PBIS is valid and usable.

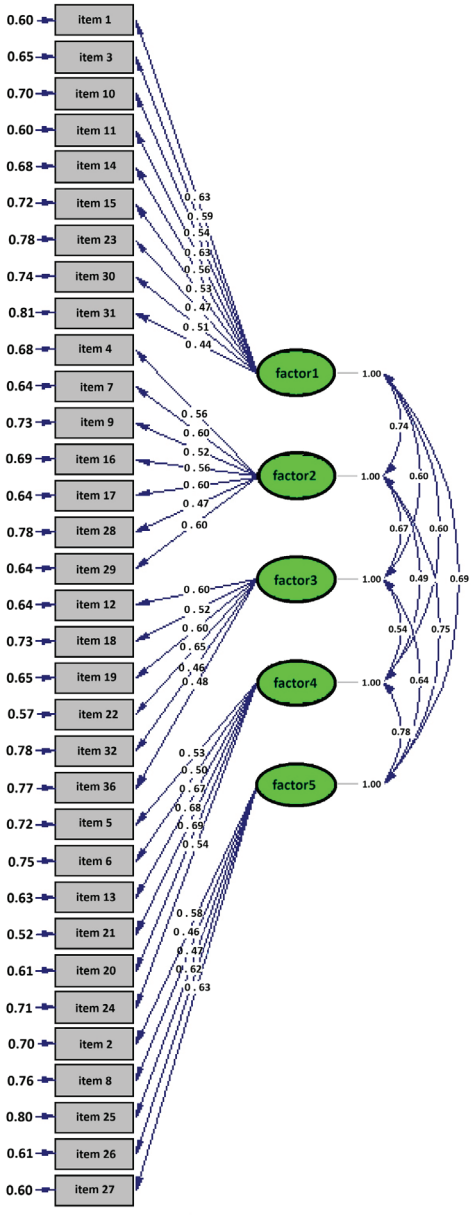

Figure 1. Confirmatory factor analysis of the Partner Breastfeeding Influence Scale

Standards other than the Cronbach's Alpha Coefficient used when evaluating reliability analysis include "item-total correlation", "mean if item deleted", and "reliability coefficient if item deleted". "Correlation analysis" is a statistical technique employed to evaluate the linear relationship between two variables and to quantify the strength of this link, if it exists. The correlation is often anticipated to be equal to or exceed 0.30 (23). In our study, the item-total correlation varied from 0.240 to 0.721 for all items, and the relationship was statistically significant ($p<0.001$). All items appear to be highly reliable and designed to assess the same variable.

Internal consistency is another criterion which indicates the reliability of a scale. Cronbach's alpha coefficient is the most favoured measure for assessing internal consistency (20,23). This method analyses whether all items in a scale exhibit a homogenous structure. This coefficient ranges between 0 and 1 (19,20,23). However, if there is a negative correlation between items, the alpha coefficient becomes negative, causing the reliability model to break down (20).

The Cronbach's alpha coefficient for PBIS was calculated to be 0.95. The Cronbach's alpha coefficient for the breastfeeding savvy subscale was 0.82, for the helping subscale, it was 0.83, for the appreciation subscale, it was 0.83, for the breastfeeding presence subscale, it was 0.83, and for the responsiveness subscale, it was 0.75. The Cronbach's alpha coefficient of the original article exceeded 0.70. These findings suggest that the scale closely resembles the original and demonstrates robust internal consistency.

Item-total correlation analysis reveals the association between the scores of individual items and the overall scores of the scale. Item-total correlation analysis is accepted as both a valid and reliable indicator (21,23). The lowest acceptable limit for item-total correlation is generally 0.20. Items with a correlation score between 0.30 and 0.40 are considered highly discriminative and reliable, while items with a correlation score higher than 0.40 are considered very highly discriminative and reliable. In our study, the item-total correlation coefficients for all 37 items in the scale ranged between 0.240 and 0.721, demonstrating statistical significance for all items. The correlation coefficients for the item-subscales were as

follows: the breastfeeding savvy subscale ranged from 0.525 to 0.741, the helping subscale was from 0.608 to 0.764, the appreciation subscale varied from 0.610 to 0.855, the breastfeeding presence subscale ranged from 0.634 to 0.778, and the responsiveness subscale had a range from 0.642 to 0.731. All values were determined to be statistically significant ($p<0.001$).

These results indicate that the item-subscale correlation of PBIS is at a sufficient level and that subscale item reliability is high.

Study Limitations

Notwithstanding its advantages, the scale possesses certain small drawbacks. This research was performed in the western region of Turkey. Despite the region's multicultural composition, this may influence the generalizability of this study's findings to the nation as a whole.

Conclusion

This study evaluated the psychometric features of the PBIS with regards to its adaptation to the Turkish language and culture. The analysis results indicated that the PBIS is both valid and reliable for the Turkish population. The PBIS is a valid and reliable instrument suitable for research projects. Comprising 37 items and 5 subscales (breastfeeding savvy, helping, appreciation, breastfeeding presence, and responsiveness), this scale has strong psychometric features and high internal consistency. The PBIS is a reliable and valid tool adapted to the Turkish language with the aim of determining the effects of fathers' behaviours on mothers in supporting breastfeeding. Using this scale for cross-sectional studies is highly recommended.

Ethics

Ethics Committee Approval: This was a cross-sectional study designed to evaluate the validation and reliability of the Turkish adaptation of the PBIS. This study received approval from the Non-Interventional Studies Ethics Committee of Manisa Celal Bayar University (approval no.: 20.478.486, date: 23.05.2018).

Informed Consent: Informed written consent was acquired from each father who consented to participate after reviewing the consent form. During the examination of their infant, the fathers who consented to participate filled out the study forms.

Acknowledgments: We extend our gratitude to all the fathers who took part in the questionnaire.

Footnotes

Authorship Contributions

Surgical and Medical Practices: E.B., N.A.D., Concept: E.B., N.A.D., Design: E.B., N.A.D., Data Collection or Processing: E.B., Analysis or Interpretation: E.B., N.A.D., Literature Search: E.B., N.A.D., Writing: E.B., N.A.D.

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

Financial Disclosure: The authors declared that this study received no financial support.

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J Pediatr Res 2024;11(4):241-249
DOI: 10.4274/jpr.galenos.2024.87528

Original Article

Prospective Observational Study of Sympathetic Failure as a Mechanism Associated with Bradycardia During Induction of General Anesthesia in Children with Down Syndrome

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ABSTRACT

Aim: While bradycardia in children with Down syndrome (DS) during inhalation induction of anesthesia is characteristic, its mechanism is not well understood. This study investigated sympathetic failure as a potential (and modifiable) mechanism of bradycardia.

Materials and Methods: Ninety-three children with DS and 102 typically developing (TD) children underwent inhalation induction of anesthesia. These children were monitored for sympathetic activity, exposed to sevoflurane anesthetic and were observed for the development of bradycardia. The primary outcome was sympathetic failure in the context of normoxic bradycardia within the first 300 seconds of induction. Secondary outcome measures included hypotension and parasympathetic excess.

Results: During the first 300 seconds of induction, 54 DS children became bradycardic (54/93, 58%) while 22 TD children became bradycardic (22/102, 22%). In the DS group, 23 experienced hypotension (23/80, 29%). Of those who experienced hypotension, 15 also experienced sympathetic failure (15/28, 54%).

Conclusion: More than half of children with DS undergoing inhalation anesthesia induction with sevoflurane experienced bradycardia. Bradycardia and hypotension were associated with sympathetic failure. Sympathetic activity therefore appears to be a modifiable target in the prevention of bradycardia in children with DS.

Keywords: Down syndrome, pediatric anesthesia, autonomic nervous system diseases, sevoflurane

***8th World Congress of Pediatric Cardiology and Cardiac Surgery, August 28, 2023 Society for Pediatric Anesthesia, March 31, 2023.**

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Received: 24.08.2024 **Accepted:** 06.11.2024

Introduction

The risks of mask-inducing a child with Down syndrome (DS) to begin a general anesthetic include upper airway obstruction due to midface hypoplasia, large tongue and adenoids, small nares, potential atlantoaxial instability, and hypotonia leading to poor venous access. Children with DS are at much greater risk of bradycardia (1-7). Heart rate nadir occurs, on average, at around 190 seconds of inhalation induction with sevoflurane and autonomic contributions have been reviewed (8). This bradycardia competes for the attention of the anesthetist and is of variable clinical significance, ranging from mild to asystole (6). The mechanism of bradycardia, and therefore its prevention or goal-directed treatment, is unknown. Impaired autonomic cardiac regulation in individuals with DS may contribute (5,9,10).

Autonomic mechanisms, such as parasympathetic excess or sympathetic failure, have been postulated as a cause of normoxic bradycardia in children undergoing inhalation induction with sevoflurane. This study aimed to characterize sympathetic failure as a candidate mechanism of this bradycardia. Our bradycardia hypothesis states that sympathetic failure is associated with bradycardia in children with DS. Our related hypotension hypothesis states that sympathetic failure is related to hypotension in children with DS.

Materials and Methods

We conducted a prospective, pragmatic observational age-matched cohort study. All study procedures occurred at a tertiary children's hospital following institutional review board approval. Recruitment was from January 12th, 2022 to December 12th, 2023. This study adhered to the applicable strengthening of reporting of observational studies (11) and is registered at ClinicalTrials.gov (NCT05120531). Ethical approval for this study was obtained from the Cincinnati Children's Hospital Medical Center Institutional Review Board (2021-0643). Sevoflurane exposure was left to the discretion of the anesthetist.

Potential participants were screened using the operating room schedule. Eligible subjects were included if they were one month to 8 years of age (inclusive), undergoing inhalation induction of anesthesia with sevoflurane for a clinically indicated surgery, had a legally authorized representative to provide informed consent and were typically developing (TD) or had a diagnosis of DS (only for the DS cohort). Children with DS were matched to TD children by age groups based on percentile charts by Fleming et al. (12). The groups were: Infants (age 1-12 months), toddlers (age 1-3 years), preschool (age 4-5 years) and school age (age 5-8 years). Subjects were excluded if they had a contraindication to adhesive use or were receiving heart rate-altering therapy. Table I presents demographic data for the entire study population. Our rationale for including participants younger than eight years is that young children are at higher risk of morbidity from bradycardia as cardiac output is more dependent on heart rate, and by age eight, children safely have adult heart rates (13).

The study exposure was clinically indicated doses of sevoflurane for the induction of general anesthesia. Participants underwent autonomic monitoring using the Vrije Ambulatory Monitoring System (VU-AMS) beginning in the preoperative holding area during at least 300 seconds of induction of general anesthesia. Following this endpoint, the monitor was removed, skin was assessed for damage, and the child was discharged from this study.

Data collection included demographic, clinical data, and autonomic data. The VU-AMS monitor was used to collect autonomic data prospectively (14,15). Autonomic data were analyzed using the Vrije Universiteit-Data Analysis and Management Software (Amsterdam, Netherlands). As the usual pre-ejection period (PEP) and respiratory sinus arrhythmia (RSA) parameters are undocumented in children with DS, each child's preoperative value was used as his or her baseline and the change for induction was computed. The deprivation index, a surrogate for socioeconomic status, was obtained by geocoding (16). Each data source was stored securely in REDCap® and de-identified using subject numbers (17,18).

The primary outcome is normoxic bradycardia within the first 300 seconds of induction. Those children who experience normoxic bradycardia during the induction of anesthesia were labeled as chronotropic incompetence due to sympathetic failure if the bradycardia occurred with a 10% or greater lengthening (increase) of the PEP compared to the preoperative measurement. Normoxic bradycardia, from now on called bradycardia, is defined as a heart rate less than the 10th percentile for age for children during the first 300 seconds of general anesthesia under normoxic (SpO_2 >90%) (12). Bradycardia thresholds for the infants age group are below 115 beats per minute, toddlers below 98, preschool age below 86, and school-age children below 70 beats per minute. Normoxia is defined as SpO_2 greater than 90%, measured by pulse oximetry. Ninety percent was chosen for the cut-off point as children rarely become bradycardic due to hypoxia when their oxygen saturation is above 90% (19).

The secondary outcome was hypotension. Hypotension is defined as systolic blood pressure one standard deviation (SD) below normal values for age in children under general anesthesia (20) while understanding that children with DS may safely experience lower blood pressure due to their DS status and short stature (21,22). Using this liberal definition, we expected that 16% of participants would meet our definition of hypotension (i.e., one SD).

Covariates comprised age for heart rate and age and sex for blood pressure. These confines were incorporated into our definitions of bradycardia and hypotension. Potential confounders to bradycardia status might include obstructive sleep apnea, nil per os duration, sevoflurane exposure, paralytics, and/or perioperative heart rate altering medications.

Autonomic parameters, PEP and RSA, were acquired. Impedance cardiogram scoring was performed manually by one author (JWS) according to published guidelines (23). PEP reflects sympathetic nervous system activity. Lower values of PEP indicate sympathetic stimulation, i.e., a shorter time in milliseconds from onset of ventricular depolarization to aortic valve opening. Given that parasympathetic excess has been posited to cause bradycardia, parasympathetic activity was also measured via RSA. RSA equals the longest inspiratory R-R interval minus the shortest expiratory R-R interval and is measured in milliseconds (24-26). RSA analysis ideally comprises at least four minutes or 240 seconds of continuous data. Therefore 300 seconds of data are required for this timing to fulfill both requirements.

Table I provides a demographic comparison between those participants with and those without DS. The presence of DS confers the development of bradycardia with an odds ratio of 9.56 (1). If we attribute all bradycardic episodes to autonomic dysfunction, we conservatively assumed that 6.8-9% (5) of controls and 25% of children with DS (5) will become bradycardic. With a total sample size of approximately 156 children, we would achieve 80% power to detect a difference in autonomic activity between the groups of greater than 19%. We had planned to enroll approximately 100 children with DS and 100 without DS to account for technical failures and data loss.

Statistical Analysis

Descriptive statistics included medians with interquartile ranges or means with SDs for continuous variables and frequencies with percentages for categorical variables. In bivariate analysis, associations were tested using the Wilcoxon rank-sum test or t-test, and the chi-square or Fisher's exact test depending on variable type and distribution. Bivariate associations were also tested using simple logistic regression with or without Firth's bias reduced correction and an odds ratio with 95% confidence intervals. Statistical analysis was performed using RStudio version 2021.09.0 Build 351 (27). The sample size calculation was performed using PASS 15.0.13, release date 2/10/2020 (NCSS, LLC, Kaysville UT) (28).

Subjects were removed from this analysis if data were missing for the primary outcome, heart rate nadir or PEP. Excluded subjects had more frequent ASA 2 status and less frequent ASA 3 status, but no difference in ASA 1 or ASA 4 statuses or no other differences in demographic information. Missing data disqualified that participant from analysis in the field for which the data was missing. Missing values for oxygen saturation, and SpO2 data, were assumed to be non-hypoxic (≥90%).

Results

Two hundred forty-nine children met the inclusion criteria, of whom 195 comprised the final population. Fifty-four children were excluded as shown in Figure 1.

For the 195 participants with data for the primary outcome (93 DS, 102 TD), heart rate and PEP measurements were made for 300 seconds prior to induction (baseline) and during the first 300 seconds of anesthesia. Baseline PEP and RSA are consistent with the published normative values (26). Patients varied from one month to eight years and weighed from 2.8 to 48 kg. Baseline heart rates, systolic blood pressures, pre-ejection periods and respiratory sinus arrhythmias of the participants were no different regardless of their DS status (Table I). Each participant for whom a previous electrocardiogram was available was in normal sinus rhythm (including one participant whose pacemaker was set to DDD during the electrocardiogram and consent). The demographic information for those participants with DS stratified by bradycardia are listed in Table II.

Normoxic bradycardia in the setting of sympathetic failure was the primary outcome of this study. Bradycardia occurred with a frequency of 54/93 (58%) for children with DS and 22/102 (22%) for TD children. Preoperative PEP was similar for children with DS and for TD children (70, and 66 respectively, p=0.176). Following exposure to sevoflurane, PEP for children with DS was greater than for TD children (75 and 69 respectively, p=0.009). Consistent with PEP findings, sympathetic failure occurred in 30/93 (32%) children with DS and 29/102 (28%) TD. The specificity of sympathetic failure to predict bradycardia was 90% in children with

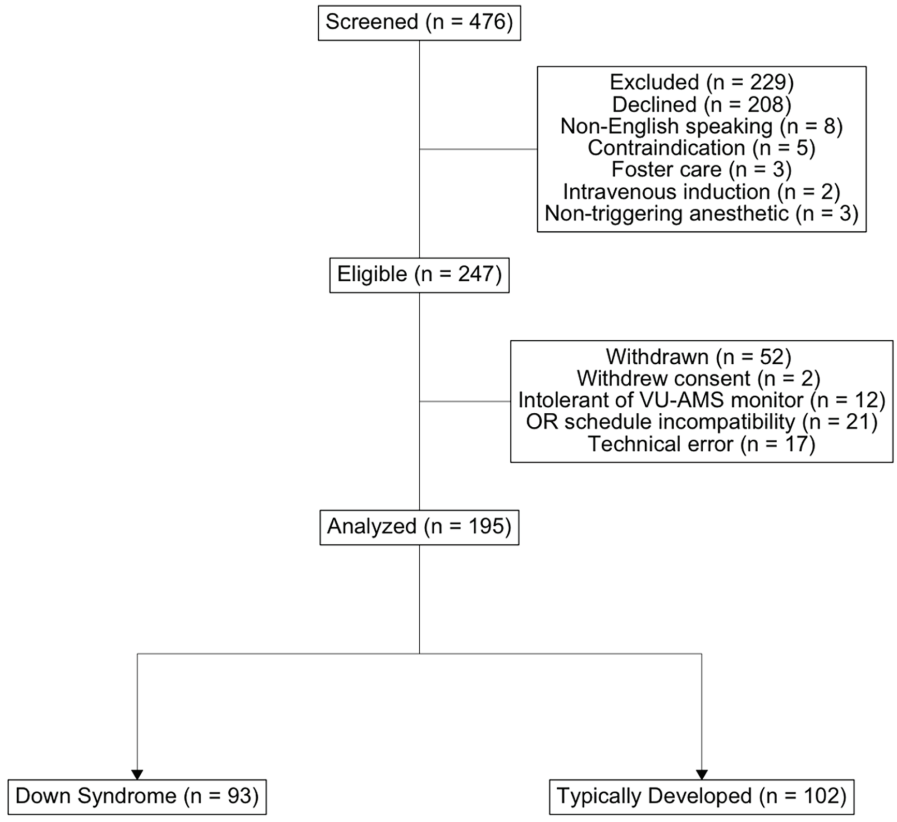

Figure 1. Participant flow through the study
VU-AMS: Vrije Ambulatory Monitoring System

DS and 76% in TD children and sympathetic failure was not sensitive in either DS or TD (48%, 45% respectively). Figure 2 shows Poincare plots of RR interval pre-induction and during induction. There were no statistically significant differences between the pre-induction and induction SD1 values for the DS children (p=0.466), or between the induction SD1 values for the DS vs TD children (p=0.329). Within the DS group, there were no differences in SD1 either pre- or during induction and bradycardia status.

The frequency of bradycardia among children with DS was (54/93, 58%) and the frequency of sympathetic failure was (30/93, 32%). We found that PEP increased significantly more in those children with DS who became bradycardic (6 milliseconds versus 1, p=0.003) than in those children with DS who did not become bradycardic. This association did not exist in TD children (p=0.083).

Factors associated with bradycardia included sympathetic failure (at least a 10% increase in PEP from baseline to induction), systolic hypotension, and the severity of hypotension. There was no difference in intraoperative administration of fentanyl, ondansetron, ketorolac, morphine, or hydromorphone among those children with DS based on bradycardia status.

Systolic hypotension occurred in 27/93 (29%) children with DS and in 9/102 (9%) TD children. Among those children with DS, bradycardia was associated with hypotension (p=0.003); in TD children, there was no association (p=0.401). In those children with DS, sympathetic failure was associated with hypotension (p=0.008). In contrast, for TD children, sympathetic failure was less common (29/102, 28%) and was not associated with hypotension (p=0.711).

We found associations between parasympathetic excess (an increase in RSA from baseline to induction) and bradycardia but not hypotension or sympathetic failure (p-values were 0.005, 0.94, and 0.14 respectively). Figure 3 shows a Venn diagram of children with DS who became bradycardic. Parasympathetic excess alone was associated with bradycardia in twenty percent of the study population (Figure 3). The independence of parasympathetic excess and sympathetic failure remains unproven. Figure 3 shows 15 children with DS and bradycardia with simultaneous parasympathetic excess and sympathetic failure supporting

Table I. Demographic information for DANSIB participants

Characteristic	n	Overall, n=195	DS, n=93	No DS, n=102	p value
Age	195	3.00 (1.00, 5.00)	3.00 (1.00, 5.00)	2.50 (1.00, 4.00)	0.410
Male gender	195	123 (63%)	64 (69%)	59 (58%)	0.113
Weight	195	13 (10, 18)	13 (10, 17)	14 (10, 20)	0.055
Non-white race	195	31 (16%)	19 (20%)	12 (12%)	0.098
ASA PS	195				**<0.001**
1		26 (13%)	0 (0%)	26 (25%)	
2		66 (34%)	11 (12%)	55 (54%)	
3		99 (51%)	79 (85%)	20 (20%)	
4		4 (2.1%)	3 (3.2%)	1 (1.0%)	
Hispanic ethnicity		4 (2.1%)	3 (3.2%)	1 (1.0%)	0.349
Public insurance		78 (40%)	32 (34%)	46 (45%)	0.128
Congenital heart surgery	113	22 (19%)	21 (29%)	1 (2.4%)	**<0.001**
Missing		82	21	61	
Hypothyroidism	195	21 (11%)	20 (22%)	1 (1%)	**<0.001**
OSA	195	57 (29%)	47 (51%)	10 (9.8%)	**<0.001**
Deprivation index	178	0.31 (0.26, 0.42)	0.30 (0.25, 0.39)	0.34 (0.26, 0.43)	0.279
Missing		17	12	5	
Baseline HR	195	113.6 (19.29)	112.6 (19.6)	114.6 (19.04)	0.480
Baseline systolic BP	133	103.5 (13.43)	103.0 (15.64)	104 (11.32)	0.696
Missing		62	32	30	
Baseline PEP	195	68 (60, 77)	70 (61, 80)	66 (60, 74)	0.176
Baseline RSA	195	39 (25, 65)	41 (29, 63)	37 (25, 65)	0.352
Sevoflurane exposure	195	4.56 (3.61, 5.41)	3.70 (3.06, 4.30)	5.28 (4.67, 5.62)	**<0.001**

DANSIB: Down syndrome autonomic nervous system induction bradycardia, ASA PS: American Society of Anesthesiologists' Physical Status, OSA: Obstructive sleep apnea, Deprivation index: Material community deprivation (2019 Brokamp), Preop dexmedetomidine: Preoperative (sedative) intranasal dexmedetomidine, HR: Heart rate, BP: Blood pressure, PEP: Pre-ejection period, RSA: Respiratory sinus arrhythmia

a modern understanding of autonomic physiology. Systolic hypotension and/or sympathetic failure was present in 33 of the 54 (61%) instances of bradycardia. The 10 children with intact sympathetic and parasympathetic activity and who maintained their blood pressures spanned all age groups: Infants (2), toddlers (3), preschool (3), and school age (2), which is in contradiction to the idea that the physiology of bradycardia and hypotension relates to chronological age.

Sevoflurane exposure was lower among DS and yet bradycardia was more common. Figure 4 shows sevoflurane exposure over each of the five minutes of induction. There was no significant correlation with heart rate for end tidal sevoflurane during any minute.

Those factors anticipated to impact bradycardia and/or sympathetic failure included nil per os time for clear liquids, preoperative heart rate altering medications, sevoflurane exposure, and obstructive sleep apnea.

Preoperative albuterol was given to 6 DS and 4 TD individuals. Obstructive sleep apnea (OSA) was present in 47/93 (51%) of DS patients and 10/201 (9.8%) of TD children. We found no relationship between OSA and bradycardia.

Discussion

Our objective was to evaluate sympathetic failure as a mechanism for bradycardia during inhalation induction with sevoflurane. We found an association between sympathetic failure and bradycardia in children with DS but not in TD children. Unlike our findings, another observational study of children with a mean age of 8.6 years found no association of hypotension with bradycardia in children with DS (29). However, our participants had a mean age of 3.5 years,

Table II. Comparison by bradycardia or not among children with DS

Characteristic	n	Overall, n=93	No, n=39	Yes, n=54	p value
Age	93	3.00 (1.00, 5.00)	3.00 (0.00, 5.00)	3.00 (1.00, 4.00)	0.651
Male gender	93	64 (69%)	26 (67%)	38 (70%)	0.704
Weight (kg)	93	13 (10, 17)	13 (7, 17)	12 (10, 17)	0.843
Non-white race	93	19 (20%)	12 (31%)	7 (13%)	**0.036**
ASA PS	93				0.240
2		11 (12%)	2 (5.1%)	9 (17%)	
3		79 (85%)	36 (92%)	43 (80%)	
4		3 (3.2%)	1 (2.6%)	2 (3.7%)	
Hispanic ethnicity	93	3 (3.2%)	2 (5.1%)	1 (1.9%)	0.570
Public insurance	93	32 (34%)	11 (28%)	21 (39%)	0.285
Deprivation index	81	0.30 (0.25, 0.39)	0.31 (0.26, 0.41)	0.30 (0.25, 0.37)	0.624
Missing		12	8	4	
Prior cardiac surgery	93	21 (23%)	7 (18%)	14 (26%)	0.364
Hypothyroidism	93	20 (22%)	9 (23%)	11 (20%)	0.754
Preoperative midazolam	93	12 (13%)	8 (21%)	4 (7.4%)	0.063
OSA	93	47 (51%)	19 (49%)	28 (52%)	0.765
Documented PCP	93	90 (97%)	37 (95%)	53 (98%)	0.570
Preoperative dex	21	2 (9.5%)	1 (7.7%)	1 (13%)	>0.999
Missing		72	26	46	
Preoperative albuterol	21	6 (29%)	3 (23%)	3 (38%)	0.631
Missing		72	26	46	
PEP baseline (msec)	93	70 (61, 80)	74 (59, 81)	68 (61, 78)	0.624
RSA baseline (msec)	93	41 (29, 63)	38 (21, 57)	46 (32, 72)	0.066
Heart rate baseline	93	112.6 (19.61)	116.7 (23.67)	109.7 (15.64)	0.110
Mean end tidal sevoflurane	93	3.70 (3.06, 4.30)	3.90 (3.12, 4.91)	3.66 (3.02, 4.03)	0.108
1st minute sevoflurane	84	3.70 (2.50, 4.80)	3.55 (1.90, 4.60)	3.75 (2.65, 4.90)	0.483
Missing		9	3	6	
2nd minute sevoflurane	79	4.00 (3.15, 4.95)	4.25 (3.43, 5.20)	3.80 (3.00, 4.70)	0.191
Missing		14	9	5	
3rd minute sevoflurane	86	4.10 (2.83, 4.78)	4.40 (3.03, 5.33)	4.00 (2.83, 4.60)	0.164
Missing		7	3	4	
4th minute sevoflurane	84	3.55 (2.90, 4.70)	4.35 (3.18, 5.10)	3.40 (2.75, 4.10)	**0.013**
Missing		9	3	6	
5th minute sevoflurane	89	3.20 (2.40, 4.20)	3.80 (2.83, 4.60)	2.90 (2.25, 3.70)	**0.005**
Missing		4	1	3	
Blood pressure baseline documented	92	61 (66%)	26 (68%)	35 (65%)	0.719
Systolic baseline	61	103.0 (15.64)	104.4 (14.39)	102.0 (16.63)	0.541
Missing		32	13	19	
Intra-op PIV placed	93	84 (90%)	32 (82%)	52 (96%)	**0.032**

Table II. Continued

Characteristic	n	Overall, n=93	No, n=39	Yes, n=54	p value
High-risk PRAP	13	1 (7.7%)	1 (17%)	0 (0%)	0.462
Missing		80	33	47	
Intra-op muscle relaxation	93	12 (13%)	7 (18%)	5 (9.3%)	0.217
Intra-op propofol admin	93	44 (47%)	23 (59%)	21 (39%)	0.056
Int propofol mg/kg dose	44	1.45 (1.09, 2.07)	1.32 (0.98, 1.82)	1.58 (1.22, 2.08)	0.290
Missing		49	16	33	
PEP induction	93	75 (64, 84)	74 (60, 83)	76 (67, 86)	0.129
Delta PEP	93	3 (0, 10)	1 (-2, 4)	6 (1, 15)	**0.003**
Systolic hypotension	93	27 (29%)	5 (13%)	22 (41%)	**0.003**
Severity of hypotension	93	7 (-2, 15)	13 (8, 23)	3 (-8, 8)	**<0.001**

ASA PS: American Society of Anesthesiologists' Physical Status, OSA: Obstructive sleep apnea, PCP: Primary care physician, Dex: Dexmedetomidine, PEP: Pre-ejection period which reflects sympathetic nervous system activity, RSA: Respiratory sinus arrhythmia which reflects parasympathetic nervous system activity, Intra-op: Intraoperative, PIV: Peripheral intravenous line placement, PRAP: Psychological risk assessment in pediatrics, Delta PEP: Change in PEP from baseline to induction, severity of hypotension - difference between systolic threshold and intraoperative systolic nadir, a greater difference indicates more severe hypotension
Descriptive statistics use n (%) for categorical variables and mean (standard deviation) if normally distributed and median (interquartile range) if not normally distributed for continuous variables

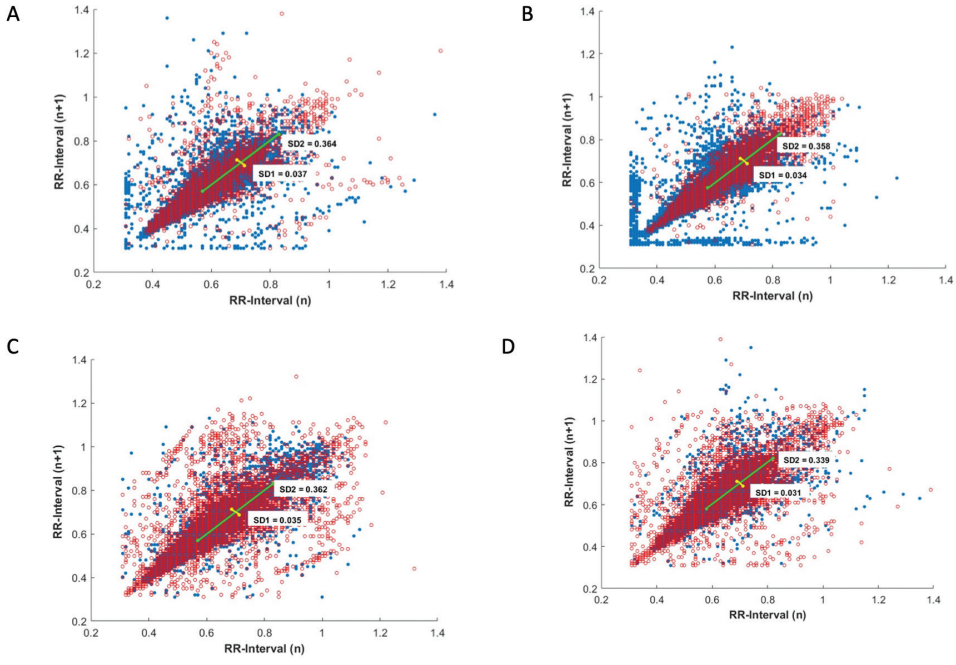

Figure 2. (Panels A and B). Poincare plots of RR Interval during induction. Children with Down syndrome, Panel A, those who became bradycardic had a larger SD1 (standard deviation perpendicular to the long axis of the plot) compared with TD children, Panel B. SD1 comprises the standard deviation of the difference between an RR interval and its predecessor, then the square root of that value. SD2 indicates the square root of the standard deviation of an RR interval
TD: Typically developing, SD: Standard deviation

and so age and heart rate dependence for cardiac output may explain this difference. A child with DS is expected to have blood pressure around the 37th percentile for age in children under five years (22). Therefore, our estimation of hypotension in children with DS may be inflated; however, there was no difference in preoperative blood pressures between the DS and TD children and so we used the same blood pressure thresholds in both groups.

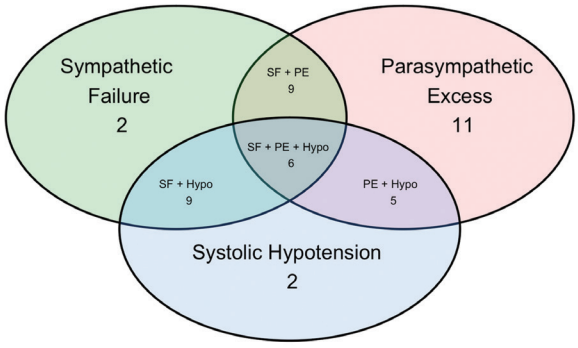

Figure 3. Venn Ddagram of children with Down syndrome who developed bradycardia during inhalation induction of anesthesia.

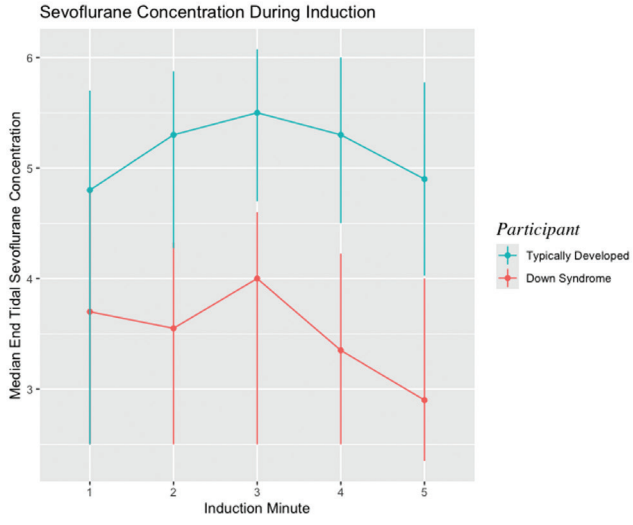

Figure 4. End tidal sevoflurane concentration over each of the first five minutes of inhalation induction in typically developed versus children with Down syndrome

The time course of development of bradycardia is rapid but not instantaneous suggesting a sympathetic (slower) rather than parasympathetic (over a single heart beat) mechanism (30).

The causation of bradycardia is unproven and likely multifactorial. Other factors include activity of the renin angiotensin aldosterone system, baroreceptor function, endocrine function and the physical pressure of the mask on the child's face (9,31). This study was the first to document separate sympathetic and parasympathetic measures of autonomic activity in young children exposed to sevoflurane, so there are no sevoflurane-exposed values for comparison. Sevoflurane has a vagolytic effect (32) and therefore should have been protective against bradycardia.

Study Limitations

The primary limitations of this study are methodological and statistical. We were unable to dictate the child's posture during baseline autonomic measurements and heart rate altering exposures such as sevoflurane. Ideally, baseline data would have been obtained with the participant supine; however, the hydrostatic pressure gradient effects of posture on cardiac output are less significant on shorter individuals such as children (21). The anesthetic technique and method of induction were not controlled. Experienced anesthetists may modify their technique of mask induction on children with DS.

Conclusion

In sevoflurane-induced bradycardia in children with DS, sympathetic failure and systolic hypotension predominate. When exposure to sevoflurane is associated with failure to maintain sympathetic tone, bradycardia and hypotension are frequent. Implications for the care of children with DS undergoing inhalation induction with sevoflurane include a search for other mechanisms to explain this phenotype. Further study of the use of a sympathomimetic agent for bradycardia prophylaxis prior to induction could be considered.

Ethics

Ethics Committee Approval: Approval for this study was obtained from the Cincinnati Children's Hospital Medical Center Institutional Review Board (2021-0643).

Informed Consent: A signed consent form was obtained from each study participant.

Acknowledgments: The authors acknowledge the support of our undergraduate student assistant, Jonathan Palmer and the Information Services for Research (IS4R) shared facility at Cincinnati Children's Hospital Medical Center (RRID:SCR_022622). IS4R services were provided in part by the National Center for Advancing Translational Sciences of the National Institutes of Health under Award Number 5UL1TR001425-03. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH. The authors also acknowledge Maria Ashton MS, RPH, MBA for providing writing assistance, editing and proofreading.

Footnotes

Authorship Contributions

Concept: J.W.S., Design: J.W.S., S.M., J.M.D., L.D., D.C., Data Collection or Processing: J.W.S., J.M., K.G., L.D., Analysis or Interpretation: J.W.S., J.M., J.M.D., L.G., Q.D., D.C., S.W., Literature Search: J.W.S., K.G., Writing: J.W.S., S.M., J.M.D., L.D., D.C., S.W.

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

Financial Disclosure: The authors declared that this study received no financial support.

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J Pediatr Res 2024;11(4):250-253
DOI: 10.4274/jpr.galenos.2024.55706

Case Report

A Novel Homozygous Variant in the *Aspartoacylase* Gene Causes Canavan Disease-Case Report

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ABSTRACT

Glu178 is the active site residue essential for substrate affinity and catalytic activity of the aspartoacylase enzyme. Sanger sequencing in an infant with Canavan disease revealed a homozygous ASPA: c.532G>A: p. (Glu178Lys) variant. Glu178Lys is the first ever variant reported at the critical active site of aspartoacylase protein and this variant might significantly disrupt substrate interaction.

Keywords: Aspartoacylase, brain, Canavan disease, mutation, myelin, N-acetylaspartate

Introduction

Canavan disease (CD) is a rare and serious autosomal recessive neurodegenerative disorder associated with spongy degeneration of the white matter of the brain. It is caused by a mutation in the aspartoacylase (*ASPA*) gene leading to the loss of or reduced ASPA enzyme activity (1,2). This disease has been reported worldwide, but is more often seen in Ashkenazi Jewish populations. The estimated incidence of severe CD in the non-Jewish population is about 1:100,000 births (3). The estimated prevalence of CD in the Arab world ranges from 1:6,000 to 1:14,000 (4). The prevalence of CD in India is not known. The ASPA protein comprises 313 amino acids with an approximate molecular weight of 36 kilo Daltons. It forms a dimer with zinc at the catalytic site and facilitates the hydrolysis of N-acetyl L-aspartate (NAA) into aspartic acid and acetate. The *ASPA* gene is located on the short arm of chromosome 17 and comprises 29 kilobases with six exons and five introns. The lack of ASPA activity leads to demyelination resulting from the accumulation of NAA in the brain. The phenotype ranges from severe typical to less severe atypical CD. Typical CD, the most common type, manifests with neurodevelopmental impairment by three to five months of age, followed by progressive neurodevelopmental regression. Atypical CD usually manifests with neurodevelopmental delay in the first years of life, followed by developmental regression in childhood or adolescence and has a more variable clinical course than typical CD. Genotype-phenotype correlations have been proposed depending on the effect of variants on the residual ASPA. Pathogenic variants p.Tyr231Ter

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Received: 09.08.2024 **Accepted:** 10.10.2024

and p.Glu285Ala, which are most commonly seen in the Ashkenazi Jewish populations, are associated with typical CD. The pathogenic variant p.Ala305Glu, commonly seen in European individuals without Ashkenazi Jewish ancestry, manifests with both typical and atypical CD. Pathogenic variants p.Arg71His, p.Asp204His, p.Pro257Arg and p.Tyr288Cys are associated with atypical CD. The usual findings on magnetic resonance imaging (MRI) studies of the brain are diffuse, symmetric white matter changes, especially in the subcortical and cortical areas. The MR spectrometry (MRS) to detect NAA has been reported as being the best method for the diagnosis of CD in infants (1). The molecular diagnosis of CD can be achieved using Sanger sequencing for the sequencing of all exons of the *ASPA* gene. Exome sequencing can also be used to identify variants in other genes which might be responsible for causing phenotypes similar to CD. Classical clinical features and elevated NAA in urine and/or with bi-allelic pathogenic variants in ASPA identified by molecular genetic testing confirm the diagnosis of CD in a proband. Currently, the treatment is mainly supportive as there is no specific treatment, and the prognosis is guarded as the majority of cases with CD die in the first decade of life (1). We report a case of novel homozygous variant at the Glu178Lys residue, a critical active site of the *ASPA* gene leading to CD.

Case Report

A 9-month-old girl, second-born to a second-degree consanguineous marriage, was admitted with a history of delay in attaining age-appropriate milestones in all domains. The mother had observed that the infant had no head control by 6 months of age. Also, the infant did not turn the head to sound nor fix and follow light. The infant recognized the mother by 7 months of age. The infant had no history of convulsions or abnormal movements. The family history was insignificant except for consanguineous marriage among the parents of the infant. The elder sibling, a seven-year-old male, did not have any symptoms of the disease. The infant had an uneventful perinatal history. On examination, her weight, length, and head circumference were 5.7 kg (<-3SD), 65 cm (<-2SD) and 47 cm (>+2SD) respectively. She had macrocephaly (Figure 1) with an open anterior fontanelle (2x2 cm). Her developmental assessment revealed global developmental delay. She had no head control, social smile or visual fixation. She could not hold objects in her hand. The infant inconsistently turned her head to sound. The infant had generalized hypotonia (central more than peripheral) and intermittent hypertonia in all four limbs. A detailed ophthalmological examination revealed bilateral optic atrophy. Hearing assessment with distortion product oto-acoustic emissions revealed bilateral presence of emissions. Brainstem evoked response audiometry could not be carried out as the test was not available at our centre. The MRI of the brain revealed flair and T2 hyper intensities in the internal capsule, subcortical fibers, and bilateral cerebral and cerebellar hemispheres. The MRS demonstrated markedly elevated NAA. Molecular analysis was performed using DNA extracted from the blood samples of the proband and her mother, obtained after informed consent, followed by amplification of all exons of the *ASPA* gene. Sanger sequencing of the amplified exons revealed a heterozygous ASPA: c.532G>A:p..-(Glu178Lys) variant in the exon-4 region (Figure 2a) in the mother, consistent with carrier status, and a homozygous variant in the proband aligning with an autosomal recessive inheritance. Sanger sequencing was not performed on the father as he did not consent to undergo the test. Molecular docking using Auto Dock Vina revealed distinct interaction profiles for the wild type and Glu 178Lys variant of ASPA with substrate AS9 (5,6). 2D and 3D interaction maps show that the wild type engages robustly through hydrogen bonds and ionic interactions centered around Glu178, supporting a strong binding affinity of -6.3 kcal/mol. In contrast, the Glu178Lys variant, with a reduced affinity of -5.7 kcal/mol, displays an altered binding landscape with fewer hydrogen bonds and increased hydrophobic contacts, indicating the variant's destabilizing effect on substrate binding (Figure 2b). A final diagnosis of CD was made, and the parents were provided

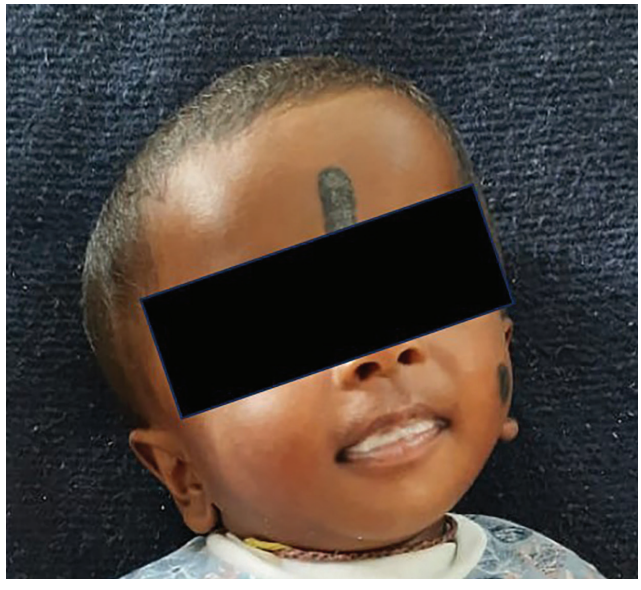

Figure 1. Macrocephaly in the infant

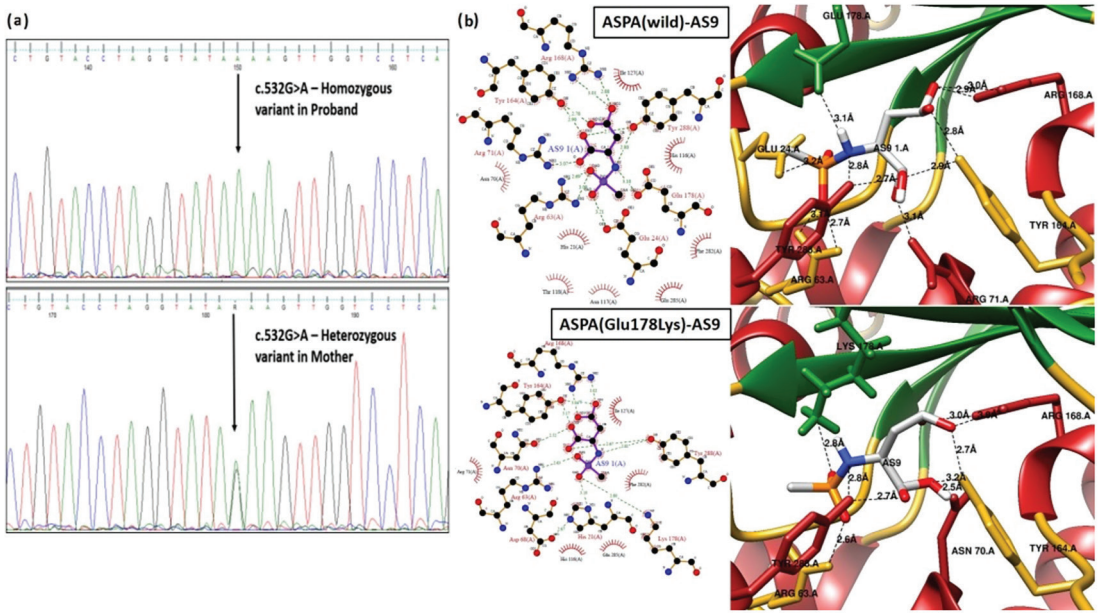

Figure 2. Genetic and molecular interactions in ASPA. (a) Sanger sequencing chromatograms displaying the c.532G>A variant in ASPA gene (b) Comparative molecular interaction maps of *ASPA*-wild type (top panel) and ASPA-Glu178Lys variant (bottom panel) with substrate AS9, including 2D interaction diagrams (left) and 3D representations (right)
ASPA: Aspartoacylase

genetic counseling. The infant was registered at the district early intervention center in our institute and provided with supportive care. The infant was lost to follow-up.

Discussion

Infants with severe forms of CD usually manifest with hypotonia, macrocephaly, and developmental delay by three to five months (1). The usual MRI findings include diffuse, symmetrical white matter changes predominantly in the subcortical and cortical areas. It is reported that detection of NAA by MRS is the prime method for the diagnosis of CD in infants (1). This infant had the classical clinical and radiological features suggestive of CD.

The ASPA protein, pivotal in CD pathogenesis, displays two-domain architecture. The N-terminal domain is structurally composed of a six-stranded β-bundle surrounded by eight α-helices, while the C-terminal domain predominantly consists of β-sheet and coil structures wrapping around the N-terminal domain. The ASPA protein family is characterized by conservation of four identical residues Asn70, Asp114, His116, and Glu178 across the 33 seed members, in addition to other conserved residues such as His21, Gly22, Glu24, Asn54, Arg63, Arg71, and Phe73 located near the active site (7). Notably, the active site, critical for its enzymatic function, is constituted by residues from the N-terminal domain with a particular emphasis on the conserved Glu178. This residue acts as a general base to activate a nucleophilic water molecule coordinated to the zinc ion, thereby facilitating the release of the L-aspartate product from the enzyme (8).

The gene coding for ASPA has been mapped to chromosome 17p13-ter (2). More than 70 different human *ASPA* gene mutations have been reported (9). The majority are missense mutations located remotely from the catalytic site. About 60% of the known missense mutations are located within the N-domain. A study (10) observed c.162 C>A (p.Asn54Lys), c.859 G>A (p.Ala287Thr), c.728 T>G (p.Ile243Ser) and c.902 T>C (p.Leu301Pro) mutations in CD patients from the Indian subcontinent. In this case, the variant observed was ASPA: c.532G>A:p.(Glu178Lys). Gene therapy is an emerging treatment approach aimed towards curing CD (11). This case emphasizes the importance of genetic testing. Precise knowledge of the mutation type is critical in aiding tailored gene therapy approaches, potentially improving their efficacy and safety. In this case, the disparity in the binding characteristics of the variant ASPA suggests a significant alteration in substrate affinity which might contribute to the pathogenesis of CD. Glu178Lys, the first ever variant reported at the critical active site of ASPA, could potentially lead to a substantial reduction in substrate binding efficiency, reducing overall enzymatic function. We report a novel variant at the Glu178Lys residue of ASPA, which could expand the opportunity for tailored treatments and therapies in CD.

Ethics

Informed Consent: Molecular analysis was performed using DNA extracted from the blood samples of the proband and her mother, obtained after informed consent, followed by amplification of all exons of the *ASPA* gene. Written informed consent has beenobtained from the parents of the child for publishing this case report.

Footnotes

Authorship Contributions

Surgical and Medical Practices: A.V.K., S.K.L., D.K., K.S., K.R.Y., M.K., T.P.L., A.D., Concept: S.K.L., Literature Search: A.V.K., S.K.L., D.K., K.S., K.R.Y., M.K., T.P.L., A.D., Writing: A.V.K., S.K.L., D.K., K.S., K.R.Y., M.K., T.P.L., A.D.

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

Financial Disclosure: The author(s) acknowledge funding support from Department of Biotechnology, Ministry of Science and Technology, Government of India, Grant/Award Number: BT/AAQ/01/CDFD Flagship/2019 for the work featured in this manuscript.

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J Pediatr Res 2024;11(4):254-258
DOI: 10.4274/jpr.galenos.2024.54781

Case Report

Status Dystonicus: A Rare and Underdiagnosed Complication of Dystonia

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ABSTRACT

Dystonia is characterized by sustained muscle contractions which produce repetitive twisting movements or abnormal postures. If it is associated with intense frequent episodes refractory to standard drug therapy and requires urgent hospital management, it is known as status dystonicus (SD). Pediatric SD remains underdiagnosed and is a potentially life-threatening crisis. SD is very painful and uncomfortable to the patient and very much distressing to the care givers. Complications of SD include bulbar weakness compromising the upper airway, pulmonary aspiration, respiratory failure, metabolic derangements such as rhabdomyolysis, myoglobinuria, dehydration, acute renal failure and hyperpyrexia. The efficacy of medical management of SD is only 10% and mortality remains at about 10%. We report on 2 dystonic cerebral palsy children who presented to us with SD: one in respiratory failure who needed immediate intubation and a second case who was under diagnosed at admission, later diagnosed during an intensive care unit stay who partially responded to midazolam infusion and polypharmacotheraphy.

Keywords: Dystonia, status dystonicus (SD), complications, midazolam, rhabdomyolysis

Introduction

Dystonia is characterized by involuntary sustained or intermittent muscle contractions causing repetitive twisting movements, abnormal postures, or both (1,2). Status dystonicus (SD) is a severe episode of dystonia, characterized by the development of increasingly frequent or continuous severe episodes of generalized dystonic spasms refractory to standard drug therapy which necessitate urgent hospital admission (3,4). SD, which is also known as dystonic storm, is infrequently reported in children (5). Pediatric SD remains underdiagnosed, is potentially fatal, needs prolonged intensive care unit (ICU) stays and has a mortality rate of 10% (1,5,6). We report on 2 cerebral palsy (CP) children who presented with SD; one with respiratory failure who needed immediate intubation and a second case who was underdiagnosed at admission, and later diagnosed as SD who partially responded to midazolam infusion and polytherapy.

Case Reports

Case 1

A 4-year and 10-month-old female child born to non-consanguineous parents at term gestation with bilirubin encephalopathy, diagnosed with dystonic quadriplegic CP with Gross Motor Function Classification System (GMFCS) level 5 presented with fever and cough over the prior 6 days and convulsions over the prior 2 hours before admission. The convulsions were tonic posturing of the limbs and opisthotonic posturing lasting for 10 minutes, accompanied by moaning and biting of the lips. On examination, the following vitals were recorded: Temp: 106 °F, PR 128/min,

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Received: 24.08.2024 **Accepted:** 04.11.2024

RR 20/min, BP 98/44 mmHg and capillary filling time 3 seconds. The patient was pale with dental caries in the right lower molar region. The child's weight was 12.6 kg and head circumference was 45 cm (<3rd percentile). Central nervous system examination revealed hypertonia of all four limbs with brisk tendon reflexes and extension of the neck with excessive irritability. The patient had been on tablet form gabapentin and tablet form tetrabenazine for 1 year for dystonia. The child was given one dose of IV midazolam. A provisional diagnosis of dystonic CP with seizure disorder was made. The patient was started on IV Fluids, Ceftriaxone injection, amikacin injection, tablet form doxycycline and fosphenytoin injection. The investigations are shown in Table I. On the second day, the child was febrile with intermittent dystonia. Tablet form clonazepam was added. On the fourth day, there were no seizures/posturing but the patient had persistent fever. On the 6th day, the child had swelling of the left thigh without evidence of cellulitis. By the 10th day, the patient remained afebrile for 72 hours and was started on Ryle's tube feeding, and was slowly transitioned to oral feeds. On day 14, the patient had one high fever spike with increasing dystonic posturing, irritability with inconsolable crying and acute distension of the urinary bladder. Dystonic storm was suspected and we initiated IV midazolam infusion (1 microgram/kg/hour), increased the dose of fosphenytoin, with tetrabenazine tablet form, gabapentin tablet form and baclofen tablet form added. The bladder was catheterized and one liter of urine was drained. On the 15th day, on midazolam infusion, the patient's dystonic movements improved, and tube feeding was restarted. On the 17th day, spontaneous eye opening was present, on midazolam infusion, no abnormal posturing/dystonia was seen.

Case 2

A 4-year and 4-month-old male child born to non-consanguineous parents at term gestation with perinatal asphyxia and bilirubin encephalopathy, diagnosed as mixed quadriplegic CP with GMFCS level 5 presented with multiple episodic tonic movements of the bilateral upper & lower limbs over the prior 4 days. Each episode lasted 20 to 30 seconds along with arching of neck, associated with excessive crying. There was also the presence of multiple tongue bites. One day prior, he had had a fracture of the left neck femur. There was history of multiple (8 times) clavicular fractures and a right ankle fracture over the prior 2 years. He had received syrup phenobarbitone for the first 2 years of life. Subsequently, he was on tablet form baclofen for 2 years and then switched over to Ayurveda treatment 15 days prior to admission. At admission, he had multiple tongue bites with bleeding, his Glasgow coma scale was 3/15 with poor respiratory efforts, therefore, the child was intubated and put on a ventilator. His vitals were measured as follows: Temperature 103 °F, PR 172/minutes with feeble pulse volume & cold peripheries. Only a systolic BP of 84 was recordable. His capillary blood sugar (CBG) was 65 mg/dL. He received 20 mL/kg of normal saline and 2 mL/kg 10% dextrose. After 20 minutes, his CBG was 120 mg/dL and BP 88/40 mmHg. The child was started on injections of fosphenytoin and ceftriaxone with a paracetamol suppository. His ankle, knee, biceps and triceps reflexes were brisk. His investigations are shown in Table II. He was on ventilator support and had high fever spikes (Temp 105 °F not responding to paracetamol). He developed hypotension again and was started on dopamine infusion at 10 micro/kg/minute. Hypernatremia correction was started with N/2 saline. A Thomas splint was applied for the femur fracture. He had acute kidney injury (AKI) and rhabdomyolysis for which conservative treatment was given. The following day, the child's guardians wanted to take the child home against medical advice.

Discussion

Secondary dystonias are the most common cause of SD and CP accounts for 59.3% of patients (7). Both of our cases were tonic type dystonic CP with a past history of bilirubin encephalopathy. SD usually occurs in known dystonia patients and ***de novo*** presentation is rare (6). Fever, infection, surgery and trauma are the frequent triggering factors (3,5). Fever was the trigger in our first child. Femur fracture and fever were the triggers in the second child. SD is a potentially life-threatening crisis which is associated with excessive exhaustion and severe pain (1). SD remains underdiagnosed in children (5). In the first case, the child was diagnosed as dystonic quadriplegic CP and had been on tablet form gabapentin, and tablet form tetrabenazine for the prior year for dystonia. The patient presented to us with tonic posturing of the limbs and opisthotonic posturing lasting for 10 minutes, which was accompanied by moaning and biting of the lips. The child was treated as status epilepticus with IV midazolam and fosphenytoin. We continued gabapentin and tetrabenazine in tablet form and added clonazepam. The patient had continuous inconsolable crying for hours, possibly due to severe pain which we could not diagnose early. We believe that the patient's condition was SD and not status epilepticus. On the 14th day, high fever spike was present along with increasing dystonic posturing, irritability with inconsolable

Table I. Haematological and biochemical investigations

	Case 1					Case 2
Investigations (normal value)	**Day 1**	**Day 3**	**Day 5**	**Day 9**	**Day 14**	**Day 1**
Hb (11-14 g/dL)	8.8	7.6		7.7		8.0
PCV (34-40%)	29	27		26		30.4
TLC (4,000-11,000 cells/mm^3)	12,230	6,970		14,400		6,200
Platelets (150,000-450,000/mm^3)	1.68	2.38		9.12		3.36
CRP (0-5 mg/L)	0.42			2.8		174
ESR (<10 mm in 1 hour)	100					100
Blood urea (12-42 mg/dL)	70	21	10			141
Serum creatinine (0.2-0.7 mg/dL)	0.83	0.57	0.42			1.65
Uric acid	5.6	1.5	0.7			15.6
Serum sodium (136-145 mEq/L)	159		133			159
Serum potassium (3.5-5 mEq/L)	3.4		4.4			4.0
Blood sugar (70-140 mg/dL)	230	129				240
AST (0-40 U/L)	739		2,618	681	556	273
ALT (0-40 U/L)	188		1,600	1,052	311	75
PT/control, INR (15 seconds/1.01)	13.2/13.3, 1.01					16.8/13.3, 1.33
aPTT/control (30 seconds/1.0)	43.8/31.8, 1.3					40.2/31.8, 1.28
Serum mg (1.5-2.6 mg/dL)	2.1				1.3	2.8
Serum phosphorous (2.5-7.7 mg/dL)	4.4					4.8
Serum calcium (ionized) (8.4-10.2 mg/dL), ionized (1.16-1.32)	8.3 [1.16]				7.5	7.3 [1.06]
CK-NAC (20-200 U/L)	>22,000		>22,000			>22,000
CK-MB (up to 4.9 ng/mL)	193					91.46
Blood lactate (4.5-19.8 mg/dL)	20					20
Troponin-T (0.0127-0.0249 ng/mL)	0.114			0.145		0.311
Vitamin D	3					-
LDH (135-214 U/L)				1132		-

PCV: Packed cell volume, TLC: Total leucocyte counts, CRP: C-reactive protein, ESR: Erythrocyte sedimentation rate, AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, PT: Prothrombin time, aPTT: Activated partial thromboplastin time, Hb: Hemoglobin, CK: Creatine kinase, NAC: N-acetyl-cystein-activated, MB: Myocardial band

crying and acute distension of the urinary bladder. At this junction, we reviewed our diagnosis. Dystonic storm was suspected and IV midazolam infusion (1 microgram/kg/hour) was started. Additionally, we increased the dose of fosphenytoin, and tetrabenazine, gabapentin and baclofen in tablet form were added. The patient also had increased levels of creatine kinase (CK)-N-acetyl-cystein-activated (more than 22,000 U/L), which may have been due to the severe generalized muscle spasms. The patient responded to our treatment. In the second case, our diagnosis was SD at admission in view of multiple episodic tonic movements of the bilateral upper & lower limbs, arching of the neck, excessive crying, multiple tongue bites and fracture of left neck femur. However, the relatives of the child wanted to take the child home against medical advice.

Complications of SD include bulbar weakness compromising the upper airway, pulmonary aspiration, respiratory failure, metabolic derangements such as rhabdomyolysis, myoglobinuria dehydration, acute renal failure and hyperpyrexia (2-4).

In SD, severe generalized muscle spasms may cause rhabdomyolysis and this may lead to acute renal failure. Significant rhabdomyolysis leads to elevated CK [usually >5 times the normal range (>1,000 IU/L)], myoglobinuria, electrolyte abnormalities, and acid-base disturbances (2).

Table II. Additional investigations

	Case 1	Case 2
CSF analysis	Cell count=3/mm^3 all lymphocytes, protein=16, sugar=95, chloride=136, culture=no growth, Gram-stain negative	
COVID RT-PCR	Negative	
ABG	-	pH=7.31, PO_2=66.6, PCO_2=24, HCO_3=11.9, Base excess=-13
H1N1 RNA PCR, Influenza A Influenza B	Negative	
Abdominal sonography	Normal diffuse edema left thigh, Doppler venous left lower limb normal	
Sonography of the thigh	Normal	
Venous doppler of the lower limbs	No thrombosis	
Other tests	Blood, CSF and stool culture sterile, Widal and Weil Felix negative Dengue NS1 and IgM negative Leptospira IgM ELISA negative Serology for HAV, HbSAg, HCV and HEV negative	Blood and stool culture sterile, Dengue NS1 negative
Femur X-ray		Left femur neck fracture

CSF: Cerebrospinal fluid, COVID: Coronavirus, ABG: Arterial blood gass, RT-PCR: Real time-polymerase chain reaction, HCV: Hepatitis C virus, Ig: Immunoglobulin, ELISA: Enzyme-linked immunosorbent assay

Both our children had CK levels >220,000 IU/L along with significant elevation of cardiac enzymes (Table I). The first case also had AKI and hypernatremia. Most likely, cardiac muscles also undergo spasms which were the cause of elevated cardiac enzymes in both cases. In SD, dystonic spasms of the upper airway and respiratory muscles result in alveolar hypoventilation and hypoxemia thereby necessitating tracheal intubation and ventilation (2,4). In our second case, SD occurred continuously for 4 days in their home which led to hypoventilation which necessitated intubation and ventilation. Hyperpyrexia occurs due to muscle spasm-induced exothermia (2). Both of the current cases had high fever spikes of 105 °F which did not respond to paracetamol. Therefore, in order to monitor these complications, SD patients should be managed in an intensive care setting (3,6).

SD should be differentiated from other disorders such as neuroleptic malignant syndrome and malignant hyperthermia (3) as those drugs used in the treatment of dystonia (tetrabenazine) (used in first child) have been implicated in the cause malignant syndrome (3,4). SD typically occurs in children. Further clues include the phenomenology of the underlying movement disorder, associated neurological symptoms and signs, a history of triggers, and time durations (2,6).

Lumsden et al. (8) proposed a Dystonia Severity Assessment Plan (DSAP) based on clinical and laboratory investigations which guide its treatment. Grade 1: The child sits comfortably with regular periods of uninterrupted sleep. Grade 2: The child is unable to sit, can only tolerate lying and able to sleep at night. Grade 3: Unable to sleep or sit comfortably, no evidence of metabolic decompensation. Grade 4: Early multi-organ failure, metabolic decompensation (e.g., acidosis, hyperkalemia, hypocalcemia, AKI, myoglobinuria, and creatinine kinase >1,000 IU/L); Grade 5: SD & multi-organ failure, requires pediatric ICU (1,8). Both of our cases were Grade 5. In a study by Goswami et al. (5), out of 23 SD children, 8 of them had CP and 50% had identifiable triggers. All of them were in DSAP 4/5 grades and needed polypharmacotherapy with >4 drugs (5).

The strategy of management includes the first 24 hours and the next 2-4 weeks (6). Intravenous midazolam is the first choice due to its muscle relaxation effect (5,6). Midazolam infusion is titrated to achieve a cessation of dystonia followed by tapering on achieving DSAP Grade 3 and a switch to an intermittent dosing schedule (5). If dystonia is not controlled, propofol is the second line drug. Third line drugs are non-depolarizing paralytic agents such as pancuronium and barbiturates (6). The duration of sedation is determined by the frequent evaluation of the child by intermittently reducing the dose of sedation. Our

first case responded to midazolam infusion. Management over the next 2-4 weeks is aimed at symptomatic dystonia control and supportive therapies. Specific dystonia treatment includes anticholinergics, dopamine receptor blockers, tetrabenazine, clonidine, baclofen and assorted drugs. A triad of drugs (the Marsden cocktail) benzhexol, tetrabenazine and pimozide may be useful (6). Our first child was on polypharmacy therapy without much benefit which led us to try Ayurveda treatment. Goswami et al. (5) observed that SD occurred in 43% children while on anti-dystonic drug therapy (5). In fact both our children were on anti-dystonic drug therapy. Deep brain stimulation can be considered early on during the first 24 hours (5,9). However financial issues and the requirement of an experienced neurosurgery team may be challenging (5). The efficacy of medical management of dystonic storm is only 10% and mortality remains at about 10% (6,8).

Conclusion

Most likely, an early identification of SD in the first case would have decreased morbidity and early pediatric consultation could have prevented mortality in the second case. There is no clear-cut demarcation between dystonia and dystonic storm and it is difficult to predict if the patient will progress to SD. Continuous monitoring by the care-givers and clinicians, and good clinical judgment is required in order to identify the life-threatening dystonic storm.

Ethics

Informed Consent: Informed consent was obtained.

Footnotes

Authorship Contributions

Concept: J.K.K., N.H.R., Design: M.V., A.A.S., Data Collection or Processing: J.K.K., N.H.R., Analysis or Interpretation: M.V., N.H.R., A.A.S., Literature Search: J.K.K., N.H.R., Writing: J.K.K., A.A.S.

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

Financial Disclosure: The authors declared that this study received no financial support.

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J Pediatr Res 2024;11(3):259
DOI: 10.4274/jpr.galenos.2024.e001

Erratum

DOI: 10.4274/jpr.galenos.2024.36937

J Pediatr Res 2024;11(2):95-102

Published in The Journal of Pediatric Research's volume 11, issue 2 of 2024 "*Aytemiz S, Sarı M, Yurttutan S, Çıkar Y, Topal S, Çaka SY. Effects of Enteral Olive Oil Supplement on Weight Gain, Length of Hospital Stay, and the Development of Some Complications in Preterm Infants: A Randomized Controlled Trial. J Pediatr Res. 2024 Jun;11(2):95-102. doi:10.4274/jpr.galenos.2024.36937*" it has been reported to us by the corresponding author that the names of two authors were inadvertently omitted. The corrections made are outlined below.

Published on page 95;

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Published on page 101;

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Corrected page 101;

(The corrected parts are given in bold)

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