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Acknowledgement

Outcomes of children with long-segment and total colon Hirschsprung disease following pull-through

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ABSTRACT

Introduction: Hirschsprung disease (HSCR) is a congenital disorder caused by the absence of ganglion cells, which leads to a functional obstruction in infants. HSCR is divided into short, long and total colon aganglionosis (TCA). However, post-operative outcome assessment of patients with long-segment and TCA is scarce. We determined the functional outcomes, Hirschsprung-associated enterocolitis (HAEC) and complications of long-segment and TCA HSCR's children following pull-through surgery.

Materials and Methods: Descriptive analysis research was done for children with HSCR long-segment and TCA who underwent an operation at our institutionfrom 2013 to 2020. We assessed the functional outcome and HAEC by the Krickenbeck and the HAEC scoring, respectively.

Results: We ascertained 13 HSCR long-segment and six TCA. We performed the following surgical procedures: Duhamel (n=7), Martin (n=4), Kimura (n=1), transabdominal Yancey-Soave (n=3) and transanal endorectal pull-through (n=4). All long-segment patients revealed good functional outcomes, whereas two TCA children suffered soiling and failed to achieve voluntary bowel movement. HAEC was noted in three long-segment and four TCA patients. Furthermore, surgical site infection and diaper rash were noticed in 10 and two patients, respectively.

Conclusion: Long-segment patients might have better functional outcomes TCA group, whereas the frequency of HAEC is compatible among arms. Long-term follow-up is important and necessary to identify complications early and define the proper treatment. Our study comprehensively analyzes functional outcomes, HAEC and complications of children with HSCR long-segment and TCA after definitive surgery in a developing country.

KEYWORDS:
Hirschsprung disease; functional outcomes; HAEC; long-segment; total colon aganglionosis

INTRODUCTION

Hirschsprung disease (HSCR) results from the incomplete development of the enteric nervous system, leading to the absence of ganglion cells in the intestines. This leads to impaired colonic function and intestinal obstruction.[1] Globally, the incidence of HSCR is approximately 1:5,000 live births,[1] with an incidence of around 1:3250 births in Indonesia.[2] Eighty percent of cases had a short segment of involvement affecting solely the rectosigmoid colon. Less commonly, extension beyond the sigmoid colon occurs (long segment, 15%), entailing the entire large bowel (total colonic aganglionosis, TCA, 5%) or, in rare cases, the complete bowel (total intestinal aganglionosis).[3]

Neonates with HSCR often present delayed meconium passage, feeding intolerance, abdominal distention and bilious emesis, indicating possible intestinal obstruction. As children advance beyond the neonatal phase, they may experience persistent constipation resistant to oral laxatives, necessitating rectal therapies. They might also exhibit symptoms such as vomiting, abdominal distention and failure to thrive.[4]

The surgical treatment for HSCR involves a pull-through procedure to remove the aganglionic colon segment and connect the ganglionized part to the anus with a functional sphincter.[5] Despite favourable outcomes after surgery, complications such as Hirschsprung-associated enterocolitis (HAEC), constipation, prolapse, perianal abscess and uncontrolled defaecation can arise.[1-3] HAEC is a life-burdening condition that can occur before and after the patient performs a pull-through.[6] In addition to HAEC, other frequently encountered complications, such as diaper rash, enterocutaneous fistula and surgical site infection (SSI).[7,8]

One of the risk factors for an unfavourable post-operative outcome is the length of the aganglionic colon—the longer the aganglionosis colon, the worse the outcome.[9] Other risk factors suspected of influencing post-operative outcome were sex, age at definitive surgery, nutritional status and surgical technique. Moreover, most studies on outcomes of children with HSCR long-segment and TCA are from developed countries.[9-11] Therefore, our study determined the variables that might influence the functional outcome and complications, particularly in HSCR patients with long-segment and TCA, from a specific developing country.

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MATERIALS AND METHODS

Subjects

The study participants comprised individuals selected based on the inclusion and exclusion criteria. Specifically, inclusion criteria encompassed HSCR long-segment and total colon aganglionosis children who had undergone pull-through surgery and were under 18 years of age during the period spanning from January 2013 to August 2020 at our institution.[12] The exclusion criteria for this study were incomplete medical records and multiple operations.

Research Design

This was a retrospective study. Data were taken from medical records, including patient characteristics: type of aganglionosis, nutritional status, age at pull-through, pull-through technique and sex, as independent variables on functional outcome, HAEC and complications as the dependent variable.

The types of aganglionosis studied in this study are long-segment and TCA. The nutritional status of the patients was divided into good and poor nutrition. Poor nutrition was determined as a weight-for-age Z score < -2.[13] Pull-through procedures were grouped into Duhamel and non-Duhamel procedures (Martin, Yancey-Soave, Kimura and transanal endorectal pull-through [TEPT]). Pull-through procedures have been performed according to previous reports.[5,14] Functional outcomes after pull-through were assessed using the Krickenbeck classification, which includes voluntary bowel movement, soiling and constipation.[15] HAEC was assessed using 16 HAEC score criteria, which was used to establish a diagnosis with a score of ≥4 or ≥10.[16] Complications included SSI and diaper rash. SSI was classified according to the National Nosocomial Infections Surveillance of the Centres for Disease Control and Prevention: incisional and organ/space SSIs. This study only included incisional SSI. Diaper rash was determined as an inflammation of the skin of the diaper area, including the perianal and perineal region.

Data Analysis

The research data were analysed descriptively using frequency, which was then expressed as a percentage or using the median and interquartile range according to the form of the data presented. Meanwhile, the Chi-square or Fisher Exact test was used to analyse significant differences and relationships between nominal variables.

RESULTS

Patient's Characteristics

Table I shows the characteristics of the subjects, including sex, nutritional status before pull-through, age at pull-through and pull-through technique grouped by type of aganglionosis.

Outcome Based on Patient's Characteristics

Table II shows the assessment of patient outcomes based on patient characteristics. Functional outcome assessments could not be carried out on the entire population of the study subjects because eight patients were less than 3years old, and one patient was still having a stoma.

Association Between Outcomes and Patient Characteristics

This study found that the type of aganglionosis did not have a significant association with functional outcomes. Likewise, other factors, namely sex, nutritional status before pull-through and age at pull-through, did not have a statistically significant association with functional outcomes. The HAEC assessment with patient characteristics did not have a statistically significant association ($p>0.05$) (Table III).

DISCUSSION

In this study, there were 13 HSCR patients with long-segment aganglionosis (4%) and 6 HSCR patients with TCA (1.8%) from a total population of 328 HSCR patients who had undergone pull-throughs from 2013 to 2020. This observed lower incidence of the long-segment type and TCA is consistent with findings from previous studies, which also reported the rarity of patients exhibiting extended or comprehensive colon aganglionosis.[3,4,9] The total ratio between male and female patients in this study was 17:2. When considering the specific type of aganglionosis, the male-to-female ratio among patients with long-segment aganglionosis stood at 12:1, while the ratio for those with total colon aganglionosis (TCA) was 5:1. A previous study has indicated variability in sex ratios, attributable to discrepancies in sample sizes and sampling durations, resulting in a more diversified collection of cases. In instances of short-segment disease, a male-to-female ratio of 3:1 to 4:1 is frequently observed, while the sex bias shifts to 1:2 to 2:1 in long-segment disease cases.[4]

Our findings revealed that 23.1% of patients diagnosed with long-segmentaganglionosis exhibited poor nutritional status. In parallel, 66.7% of patients diagnosed with TCA experienced poor nutrition. This correlation was similarly observed in a previous report that 54.5% of patients with extensive colon aganglionosis were found to have poor nutrition.[17] Notably, most patients included in this study underwent temporary stoma placement as a preliminary step before definitive therapy. The rationale behind stoma placement lies in its ability to stabilise patients exhibiting severe malnutrition or alleviate significant dilation in the proximal intestine. This intervention contributes to reducing the risks associated with preoperative complications that could lead to mortality and morbidity.[5]

This study showed that patients with a long-segment type of aganglionosis mostly performed pull-throughs at 0–24 months (77%) with a median of 16 months and an interquartile range of 7 months. Then, patients with TCA type also mostly did pull-throughs at 0–24 months (66.7%) with a median of 15 months and an interquartile range of 17.75 months. As long as there are no significant comorbidities for the patient, many surgeons prefer to conduct a pull-through shortly following the diagnosis. Preoperative enterocolitis is considered to be avoided with this approach, as well as extended hospital stays and complications from prolonged use of the stoma.[5,18]

Our study shows that the Duhamel procedure is the most commonly used pull-through technique in patients with long-segment aganglionosis (46.2%). In the TCA type, the

Table I: Patient's characteristics

Characteristics	Type of HSCR aganglionosis	
	Long-segment n = 13	TCA n = 6
Sex		
Male	12 (92%)	5 (83%)
Female	1 (8%)	1 (17%)
Nutritional status before pull-through		
Good	10 (77%)	4 (67%)
Poor	3 (23%)	2 (33%)
Age at pull-through		
0–24 months	10 (77%)	4 (67%)
> 24 months	3 (23%)	2 (33%)
Pull-through technique		
Duhamel	6 (46%)	1 (17%)
non-Duhamel	7 (54%)	5 (83%)

HSCR: Hirschsprung disease

Table II: Outcome based on patient's characteristics

Characteristics	Outcome					
	Functional outcome (n = 10)		Constipation	HAEC (n = 19)		Complications (n = 19)
	VBM	Soiling		≥ 4	≥ 10	
HSCR type						
Long	7	0	0	3	2	9
TCA	1	2	0	4	1	3
Sex						
Male	7	2	0	5	2	12
Female	1	0	0	2	1	0
Nutritional status						
Good	6	1	0	6	3	10
Poor	2	1	0	1	0	2
Age at pull-through						
0–24 months	5	1	0	5	2	7
> 24 months	3	1	0	2	1	5
Pull-through technique						
Duhamel	5	0	0	3	2	5
Non-Duhamel	3	2	0	4	1	7

HAEC: Hirschsprung-associated enterocolitis

Table III: Association between outcomes and patient characteristics

Characteristics	Outcome					
	Functional outcome		Constipation	HAEC		Complications
	VBM	Soiling		≥ 4	≥ 10	
HSCR type						
p-value	0.072	0.072	NA	0.129	1.0	0.617
Odds ratio	25.0	25.0		6.67	1.10	0.44
(95% CI)	(0.75–832.99)	(0.75–832.99)		(0.79–56.22)	(0.08–15.15)	(0.06–3.24)
Gender						
p-value	1.0	1.0	NA	0.123	0.298	0.136
Odds ratio	1.0	1.0		15.0	7.50	0.09
(95% CI)	(0.03–33.32)	(0.03–33.32)		(0.60–374.82)	(0.33–173.28)	(0.004–2.15)
Nutritional status						
p-value	1.0	1.0	NA	0.603	0.53	0.305
Odds ratio	3.0	3.0		0.33	0.30	0.27
(95% CI)	(0.12–73.64)	(0.12–73.64)		(0.03–3.80)	(0.01–6.85)	(0.03–2.25)
Age at pull-through						
p-value	1.0	1.0	NA	1.0	1.0	0.125
Odds ratio	1.67	1.67		1.20	1.50	11.0
(95% CI)	(0.07–37.73)	(0.07–37.73)		(0.15–9.77)	(0.11–21.31)	(0.51–2336.2)
Pull-through technique						
p-value	0.444	0.444	NA	1.0	0.523	0.656
Odds ratio	7.86	7.86		0.67	0.23	0.56
(95% CI)	(0.28–217.12)	(0.28–217.12)		(0.09–4.54)	(0.02–3.13)	(0.07–4.14)

NA, not applicable; HAEC, Hirschsprung-associated enterocolitis.

technique most often used (50%) is the Martin procedure, which is a modification of the Duhamel procedure and 16.7% of patients underwent the unmodified Duhamel pull-through. More prospective studies are needed to establish one technique's superiority over another. Notably, all three procedures have been extensively performed worldwide, yielding comparable outcomes in substantial long-term studies.[5]

Overall, two patients experienced functional disturbances, especially in the soiling. In patients with extensive colonic aganglionosis, the pull-through procedure becomes more complex. The anastomosis level of the bowel pull-through is considered principal for faecalcontinence, regardless of whether a transanalor transabdominal surgical method is used. Moreover, extreme stretching of the anus during transanal approach, can lead todamage of external sphincter, resulting in faecal incontinence. Another aetiology that can affect the functional outcomes after definitive surgery is the twisting of the pull-through bowel.[7]

This study shows that 36.8% of patients have HAEC with a cut-off level of ≥4, and 15.8% of patients show HAEC with a cut-off level of ≥10. Decreasing the cut-off level to 4 increases the frequency of HAEC after performing a pull-through to about three times compared to the cut-off score of 10.[15] In addition, a longer colonic aganglionosis indicates a more significant reduction in the gut immune system, producing potentially pathogenic bacteria if intestinal stasis occurs.[19]

Among the 19 patients, 12 patients had complications (63.2%). Ten patients (52.6%) experienced SSI, and two patients (10.5%) had diaper rash. The risk of SSI has generally been shown to be highest following colorectal surgery. The incidence of SSI was observed to range from 1.7% to 19.2% in HSCR evaluations.[7] Meanwhile, other complications like diaper rash or perianal excoriation in patients with TCA have a high incidence because a total colectomy with an ileoanal anastomosis is usually performed. Hence, the frequency of bowel movements increases.[17]

Our study shows that the type of aganglionosis did not have a significant association with functional outcomes. Other factors, namely sex, nutritional status and age at pull-through, also did not correlate statistically with functional outcomes. Regarding age at pull-through, our findings were compatible with a recent systematic review that concluded that no beneficial impact of delaying pull-through for patients with TCA, particularly on diaper rash frequency.[8]

The HAEC frequency and patient characteristics had no statistically significant association ($p>0.05$). Moreover, the association between complications and patient characteristics was not statistically significant. Notably, the small sample size is a weakness of our study; therefore, it is necessary to use a larger sample to clarify the findings of this study.

Several novelties have been noted in our study, including: 1) we provided new data on functional outcomes of long-segment and TCA children from a developing country (vs. developed countries[9-11]); and we comprehensively analysed the functional outcomes, HAEC and complications in HSCR patients with long-segment and TCA (vs. post-operative complications[10] vs. HAEC[16,19]).

Limitations of our study should be considered during the interpretation of our findings, including small sample size, retrospective design and one-centre study. Moreover, our findings were based on overall means without accounting for other potential factors that might affect the results, including the surgeon experiences and pull-through technique preference by attending surgeons.

CONCLUSION

Long-segment patients might have better functional outcomes TCA group, whereas the frequency of HAEC is compatible among arms. Long-term follow-up is important and necessary to identify complications early and define the proper treatment. Our study comprehensively analyzes functional outcomes, HAEC and complications of children with HSCR long-segment and TCA after definitive surgery in a developing country.

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ORIGINAL ARTICLE

Association between eosinophilia and lymphocytosis with functional outcomes of patients with Hirschsprung disease following transabdominal Yancey–Soave pull-through

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ABSTRACT

Introduction: Hirschsprung disease (HSCR) is a disorder caused by the failure of neural crest migration leading to an aganglionic colon and functional obstruction. Transabdominal Yancey–Soave pull-through is one of the definitive therapies for this condition. Prognostic factors, including sex, aganglionosis type, age at definitive surgery, nutritional status, eosinophilia and lymphocytosis, might influence the outcomes of the pull-through. We evaluated the functional outcomes of HSCR patients after Yancey-Soave surgery and associated them with the prognostic factors.

Materials and Methods: The study included Hirschsprung patients aged ≥3 and <18 years who underwent Yancey-Soave surgery at our hospital. The functional outcomes were evaluated using the Krickenbeck classification to determine voluntary bowel movement (VBM), constipation and soiling.

Results: Most (82.6%) patients showed VBM, 26.1% had constipation and 4.3% suffered from soiling. Among 23 patients who received Yancey–Soave surgery, 8 (34.8%) had eosinophilia and 5 (21.7%) had lymphocytosis. However, no significant differences were observed between eosinophilia and non-eosinophilia groups for VBM (p=1.0), constipation (p= 0.621) or soiling (p=0.738). Similarly, no significant differences were found between lymphocytosis and non-lymphocytosis groups for VBM (p=1.0), constipation (p=0.545) or soiling (p=0.973). Moreover, no other prognostic factors affected the functional outcomes after Yancey–Soave surgery (p>0.05).

Conclusion: Our study shows that eosinophilia and lymphocytosis might not affect the functional outcome of patients with HSCR following Yancey-Soave surgery. In addition, sex, aganglionosis type, age at definitive surgery and nutritional status might not influence the functional outcome after definitive surgery. Further, a more extensive study is essential to clarify our findings.

KEYWORDS:
Eosinophilia; functional outcomes; Hirschsprungdisease; lymphocytosis; Yancey–Soave pull-through

INTRODUCTION

Hirschsprung disease (HSCR) is a disease characterised by the absence of ganglion cells. It is the most common cause of functional obstruction in children,[1] with an incidence of 1 in 5000 live births[2] and a total male-to-female ratio of 1:4. The incidence of HSCR in Indonesia reaches 1 in 3250 births.[3]

One of the definitive therapies for HSCR is transabdominal Yancey–Soave pull-through which is aimed at removing the aganglionic colon and pulling the normal colon down to the anus while preserving sphincter function.[4] Several postoperative outcomes, such as Hirschsprung-associated-enterocolitis (HAEC), soiling and constipation, might be found and can lead to morbidity and mortality in HSCR patients, thus requiring evaluation.[5,6] Some prognostic factors, including eosinophilia, might influence the outcomes of the pull-through; however, they show conflicting findings.[7,8] Therefore, we evaluated the functional outcomes of HSCR patients after Yancey–Soave surgery and associated them with the prognostic factors.

MATERIALS AND METHODS

Patients

Following approval by the Institutional Review Board of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada/Dr. Sardjito Hospital, Indonesia (KE/FK/110/EC/2020), we conducted a cross-sectional study involving HSCR patients ≥3 and <18 years old who underwent Yancey–Soave surgery between 2013 and 2020 in our hospital. Patients who had incomplete medical records data were excluded.

Prognostic Factors

Patients who met the criteria were classified into some characteristics, including sex (male and female), aganglionosis (short and long), age at surgery (<3 years and ≥3 years) and nutritional status (well- and undernourished). Nutritional status was divided into well- and undernourished and assessed based on weight-for-age z-score for patients aged <5 years, where undernourished was defined as a condition in which the patient's z-score value was below –2.9 For patients aged 5–18 years, nutritional status was evaluated using body mass index.[9]

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Serum Eosinophilia and Lymphocytosis
In previous studies, eosinophilia was determined by histopathology that needs rectal biopsy.[7,8] Therefore, we chose a non-invasive, more accessible and routine method to determine the association between eosinophilia and lymphocytosis and functional outcomes using their serum level.

In this study, peripheral blood samples were collected 1–8 days before definitive surgery. Eosinophilia is described as an elevation of eosinophils >0.5 ×109/L, and lymphocytosis is characterised by an increase in lymphocytes >7 $\times 10^9$/L.

Functional Outcomes
Functional outcomes were evaluated with Krickenbeck classification to determine voluntary bowel movement (VBM), constipation and soiling. Constipation is further divided into three categories, namely grade 1 (managed through dietary patterns), grade 2 (requires laxative use) and grade 3 (resistant to dietary changes and laxative use). Meanwhile, soiling is categorised into grade 1 (1–2×/week), grade 2 (every day, without social disturbances) and grade 3 (constant and causing social disturbances).[10]

Statistical Analysis
Data were presented in numbers/percentages and analysed using the Fisher Exact or Chi-square test to examine the association between prognostic factors and functional outcomes.

RESULTS

Baseline Characteristics
In this study, HSCR patients were identified based on the International Classification of Disease Tenth Revision (ICD-10) with diagnosis coding ICD-10-CM Q43.1 and Soave procedure based on the International Classification of Disease Ninth Revision with code ICD-9-CM 48.41.[11] The patient clinical characteristics are shown in Table I. Twenty-three HSCR patients had complete data for final analysis. Most patients were male (78.3%), had short-segment HSCR (91.3%) and were well nourished (65.2%) (Table I).

Functional Outcomes
Among 23 patients, most (82.6%) showed VBM, 26.1% had constipation, and 4.3% suffered from soiling (Table II).

Association Between Prognostic Factors and Functional Outcomes
Subsequently, we determined the association between the patient's characteristics, including sex, age of pull through, aganglionosis type, nutritional status, eosinophilia and lymphocytosis and functional outcomes, i.e., VBM, soiling and constipation. None of the prognostic variableswere significantly associated with the functional outcomes: VBM, soiling and constipation following Yancey–Soave pull-through (Tables III, IV and V, respectively).

DISCUSSION

Our study reveals that most HSCR patients have VBM following the Yancey-Soave pull-through. Only a few HSCR patients show abnormal VBM. Abnormal VBM might be associated with abnormalities in anal canal function and sphincter control, as well as hypomotility.[12] Abnormalities in anal canal function and sphincter control can be associated with the location of the anastomosis. An anastomosis closer to the dentate line increases the risk of abnormal VBM and incontinence as it can damage the nerve endings at the dentate line, which are essential for holding back the urge for defecation.[13,14]

Furthermore, constipation found in this study was ~26% of cases. In HSCR patients following Yancey-Soave pull-through, the incidence of constipation is relatively high, possibly due to anastomotic strictures in the muscular cuff.[15] In the Yancey-Soave pull-through, this muscular cuff remains contracted and can lead to compression and disruption of the normal colonic peristaltic movement. This becomes a cause of functional obstruction, which can result in constipation.[16]

Interestingly, only one case of soiling was noted in our study. Soiling can be caused by injuries to the sphincter muscles, damage to the anal canal, and the presence of overflow incontinence due to constipation. However, these phenomena are rarely observed in the transabdominal pull-through technique[17,18] as our study.

Mucosal eosinophilia has been investigated to be associated with the functional outcomes of HSCR patients following pull-through; however, studies reveal conflicting findings and scarce.[7,8,19] Our study failed to show the association between serum eosinophilia and lymphocytosis and functional outcomes of HSCR patients after definitive surgery. Our findings support a current study.[7] However, we utilised a different method, i.e., serum eosinophilia vs. histopathology.[7] Another novelty of our study is that we also used another variable, i.e., serum lymphocytosis vs. eosinophilia.[7,19] In addition, we chose the serum approach due to its minimal invasiveness, less harmful to patients,routine assessment in daily practice, and ease of monitoring the changes. Our study provides new evidence that eosinophilia is not significantly associated with the functional outcomes of HSCR patients after pull-through from a specific developing country.

Interestingly, a previous study analysed rectal biopsies in non-HSCR patients and found that patients with mucosal eosinophilia were at a higher risk of experiencing constipation and growth failure.[20] Furthermore, a high incidence of recurrent abdominal pain, diarrhoea, and constipation was observed in patients with eosinophilic colitis.[21] Constipation in patients with eosinophilia is suspected to occur because eosinophils disrupt gastrointestinal motility and might have a negative impact on myenteric ganglion cells. There is also evidence that mast cells and eosinophils are critical cells in the development of dysmotility by releasing granules.[20]

In contrast, one found that despite findings of eosinophil infiltration in the myenteric plexus of HSCR patients, no gastrointestinal complications were observed.[8] It is also noted that eosinophil infiltration in the myenteric plexus of HSCR patients does not lead to a poor prognosis after surgery.[8] These findings were similar to a current study showing no significant differences in postoperative outcomes, feeding issues, and stooling issues between patient groups with and without mucosal eosinophilia.[7]

Table I: Clinical characteristics of HSCR patients who underwent Yancey–Soave pull-through in our institution

Characteristic	n=23	Percentage (%)
Sex		
Male	18	78.3
Female	5	21.7
Aganglionosis type		
Long	2	8.7
Short	21	91.3
Age at surgery		
< 3 years old	11	47.8
≥ 3 years old	12	52.2
Nutritional status		
Well-nourished	15	65.2
Undernourished	8	34.8
Eosinophilia		
Yes	8	34.8
No	15	65.2
Lymphocytosis		
Yes	5	21.7
No	18	78.3

Table II: Functional outcomes in HSCR patients after Yancey-Soave pull-through in our institution

Functional outcomes	n (%)
Voluntary bowel movement (VBM)	19/23 (82.6)
Soiling	1/23 (4.3)
√ Grade 1	1 (100)
√ Grade 2	-
√ Grade 3	-
Constipation	6/23 (26.1)
√ Grade 1	2 (33.3)
√ Grade 2	3 (50)
√ Grade 3	1 (16.7)

Table III: Association between patients' characteristics and VBM in HSCR patients after Yancey–Soave pull-through

Variables	VBM		OR (95% CI)	p-value
	Yes (n, %)	No (n, %)		
Sex				
• Male	14 (77.8)	4 (22.2)	3.41 (0.16–74.44)	0.435
• Female	5 (100)	0		
Aganglionosis type				
• Long	2 (100)	0	1.06 (0.04–27.11)	0.973
• Short	18 (85.7)	3 (14.3)		
Age at surgery (yo)				
• <3	10 (90.9)	1 (9.1)	0.3 (0.03–3.43)	0.59
• ≥3	9 (75)	3 (25)		
Nutritional status				
• Well-nourished	13 (86.7)	2 (13.3)	1.08 (0.08–14.08)	1.0
• Undernourished	7 (87.5)	1 (12.5)		
Eosinophilia				
• Yes	7 (87.5)	1 (12.5)	0.57 (0.05–6.61)	1.0
• No	12 (80)	3 (20)		
Lymphocytosis				
• Yes	4 (80)	1 (20)	1.25 (0.1–15.5)	1.0
• No	15 (83.3)	3 (16.7)		

HSCR, Hirschsprung disease; VBM, voluntary bowel movement; yo, years old

Table IV: Association between patients' characteristics and constipation in HSCR patients after Yancey-Soave pull-through

Variables	Constipation		OR (95% CI)	p-value
	Yes (n, %)	No (n, %)		
Sex				
• Male	4 (22.2)	14 (77.8)	0.43 (0.05–3.52)	0.576
• Female	2 (40)	3 (60)		
Aganglionosis type				
• Long	0	2 (100)	0.48 (0.02–11.37)	0.647
• Short	6 (28.6)	15 (71.4)		
Age at surgery (yo)				
• <3	4 (36.4)	7 (63.6)	2.86 (0.41–20.14)	0.37
• ≥3	2 (16.7)	10 (83.3)		
Nutritional status				
• Well-nourished	2 (13.3)	13 (86.7)	0.15 (0.02–1.18)	0.131
• Undernourished	4 (50)	4 (50)		
Eosinophilia				
• Yes	3 (37.5)	5 (62.5)	2.4 (0.36–16.21)	0.621
• No	3 (20)	12 (80)		
Lymphocytosis				
• Yes	2 (40)	3 (60)	3.33 (0.38–29.39)	0.545
• No	3 (16.7)	15 (83.3)		

HSCR, Hirschsprung disease

Table V: Association between patients' characteristics and soiling in HSCR patients after Yancey–Soave pull-through

Variables	Soiling		OR (95% CI)	p-value
	Yes (n, %)	No (n, %)		
Sex				
• Male	1 (5.6)	17 (94.4)	0.94 (0.03–26.63)	0.973
• Female	0	5 (100)		
Aganglionosis type				
• Long	0	2 (100)	2.73 (0.09–86.93)	0.569
• Short	1 (4.8)	20 (95.2)		
Age at surgery (yo)				
• <3	0	11 (100)	0.33 (0.01–9.07)	0.515
• ≥3	1 (8.3)	11 (91.7)		
Nutritional status				
• Well-nourished	1 (6.7)	14 (93.3)	1.76 (0.06–48.2)	0.738
• Undernourished	0	8 (100)		
Eosinophilia				
• Yes	0	8 (100)	0.57 (0.02–15.58)	0.738
• No	1 (6.7)	14 (93.3)		
Lymphocytosis				
• Yes	0	5 (100)	1.07 (0.04–29.96)	0.973
• No	1 (5.6)	17 (94.4)		

HSCR, Hirschsprung disease.

To our knowledge, the association between serum lymphocytosis and HSCR patients' outcomes has not been extensively reported. One study found that lymphocyte infiltration in the myenteric plexus of HSCR patients.[8] Lymphocyte infiltration can damage the myenteric plexus, lead to aganglionosis, and result in the loss of inhibitory anorectal responses,[22] leading to symptoms of pseudoobstruction[12] and constipation.[13] Our study was unable to reveal the association between lymphocytosis and functional outcomes of HSCR patients after pull-through. These discrepancies might be due to different approaches: histopathology8 vs. serum (our study).

Our study did not find any association between length of aganglionosis, sex, nutritional status, and age at pull-through with functional outcomes of HSCR patients following pull-through, including VBM, constipation and soling (Tables III, IV and V, respectively). Most of our patients are male, short-aganglionosis, well-nourished and undergoing definitive surgery at ≥3 years old. It is postulated that female patients may have an elevated susceptibility to experiencing constipation due to hormonal factors.[15] In addition, postoperative functional problems arise, in part, from the lack of coordinated function in the aganglionic colon.[23] The lengthening of the aganglionic colon corresponds to a more proximal site of obstruction, leading to increased intraluminal pressure and consequently exacerbating the severity of dysmotility.[24] The correlation between long-segment HSCR and worse functional outcomes is conflicting.[25-28] Some studies showed that patients with long-segment HSCR had higher soiling and incontinence rates,[25-26] whereas other reports failed to reveal the association,[27,28] Notably, older HSCR patients were observed to experience more severe intestinal obstruction.[29]

The lack of significance in the results of this study might be attributed to the postoperative functional outcomes in HSCR patientsare possibly more related to mucosal eosinophilia and lymphocytosis rather than serum eosinophilia and lymphocytosis. The association between mucosal and serum eosinophilia is controversial. One study showed that no correlation was found between serum eosinophils count and mucosal eosinophils in tissue biopsies, suggesting that peripheral eosinophilia might not imply eosinophil infiltration in tissues,[30] while another revealed that serum eosinophilia was found in 35% of patients with eosinophilic colitis.[31] It is interesting and important to perform a further study to determine: 1) the association between mucosal and serum eosinophilia specifically in HSCR patients; and 2) how mucosal and serum eosinophil interact and affect each other.

Furthermore, the absence of a statistically significant association between prognostic factors and functional outcomes within this study might also be attributed to the small sample size. The limited sample size might be due to the rarity of the transabdominal pull-through Yancey-Soave at our hospital. We currently prefer performed transanal endorectal[32] and Swenson-like pull-through due to the establishment of early diagnosis of HSCR in our institution.

CONCLUSION

Our study shows that eosinophilia and lymphocytosis might not affect the functional outcome of patients with HSCR following Yancey–Soave surgery. In addition, sex, aganglionosis type, age at definitive surgery and nutritional status might not influence the functional outcome after definitive surgery. Further, a more extensive study is essential to clarify our findings.

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ORIGINAL ARTICLE

Outcomes and prognostic factors for survival of children with oesophageal atresia

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ABSTRACT

Introduction: Oesophageal atresia (EA) is a life-threatening congenital oesophageal deformity that causes considerable newborn morbidity and death. Many prognostic variables have been linked to the survival of infants with EA, although the results of the studies are still conflicting. Furthermore, studies on EA effects in developing countries still need to be included. Here, we aimed to determine the survival of children with EA and link it to prognostic variables in a particular developing country.

Materials and Methods: A cross-sectional observational retrospective study was conducted using medical records of paediatric patients with EA at our institution from January 2014 to December 2020.

Results: A total of 53 children with EA were included in the study. Log-rank analysis showed that definitive surgery and thrombocytopenia were significantly associated with the survival of children with EA, with a p-value of 0.007 and 0.002, respectively, whereas, sex, EA type, pneumonia and sepsis were not (p = 0.898, 0.919, 0.255, and 0.499, respectively). Multivariate analysis revealed that thrombocytopenia and definitive surgery were strongly associated with the survival of children with EA with a p-value of 0.014 (hazard ratio (HR) = 2.67 [95% confidence interval (CI) = 1.22–5.85]) and 0.022 (HR =0.39 [95% CI = 0.17–0.87]), respectively.

Conclusion: Our study shows that thrombocytopenia might increase mortality, while definitive surgery might be beneficial for the survival of paediatric patients with EA. It implies that definitive surgery should be performed as early as necessary to prevent further morbidity and mortality. Our study comprehensively provides the survival of children with EA and links it to prognostic variables in a particular developing country. It serves as a potential research project that can be applied to the clinical setting to help clinicians manage EA better.

KEYWORDS:
Oesophageal atresia, prognostic factors, survival, thrombocytopenia, pneumonia, definitive surgery

INTRODUCTION

Oesophageal atresia (EA) with or without tracheoesophageal fistula (TEF) is a life-threatening congenital oesophageal deformity that causes considerable newborn morbidity and death. This abnormality occurs in one in every 2,500 to one in every 4,500 live births.[1] Over time, the treatment of EA with or without TEF has improved dramatically. Early diagnosis, better newborn critical care and anaesthesia and improved surgical methods have all contributed to this progress. As a result, the survival rate of these children is around 95% in developed countries when other serious congenital abnormalities do not accompany EA.[2–4] Unfortunately, the survival rate of infants with EA, with or without TEF, is not as favourable in developing countries. Prematurity, low birth weight and severe related congenital abnormalities have a proportionally higher impact on EA morbidity and mortality in developing countries due to a lack of adequate medical facilities.[5–7]

Several studies about the prognostic variables linked to the survival of infants with EA, including those from developing countries; however, have shown conflicting results.[5,8-10] This is why we have embarked on this study intending to examine the prognostic variables associated with a better survival of infants with EA in a developing country in Southeast Asia.

MATERIALS AND METHODS

Subjects

From January 2014 to December 2020, we conducted a cross-sectional observational retrospective study utilising the medical records of children with EA at our hospital. We examined 53 infants with EA who had surgery at our institution and had the ICD X code Q.39.1.[11] Incomplete medical records were an exclusion criterion.

The study was approved by the Institutional Review Board of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada/Dr. Sardjito Hospital (KE/FK/0963/EC/2020).

Prognostic Factors

We evaluated following prognostic factors for the survival of paediatric patients with EA: sex, birth weight, gestational age, associated anomaly, EA type, sepsis, thrombocytopenia

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Table I: Haematological score in neonatal sepsis

Haematological criteria	Abnormality	Score
Immature: Total neutrophil (I/T) ratio based on age	Increased	1
Neutrophil count	Increased or decreased	1
Immature: Mature neutrophil (I/M) ratio	≥ 0.3	1
Immature neutrophil	Increased	1
Leucocyte count		
• Decreased to ≤ 5,000/mm^3		
• Increased:		
√ At birth ≥25,000/mm^3		
√ 12-24 hours ≥30,000/mm^3		
√ Day 2 ≥21,000/mm^3	Increased or decreased	1
If degenerative neutrophils are present:		
• Vacuolisation or Dohle bodies		
√ 0 if not present		
√ 1+ if ≤ 25%		
√ 2+ if 25 to 50%		
√ 3+ if 51 to 75%		
√ 4+ if ≥ 75%		
• Toxic granulation		
√ 0 if normal granulation		
√ 1+ if few toxic granulations		
√ 2+ if 50% of the neutrophils contain dark granulation		
√ 3+ if most cells contain granulation		
√ 4+ if toxic granulation blurs nuclei	≥3+	1
Platelet count	≤ 150,000/mm^3	1

and pneumonia. Gestational ages were classified as preterm, at term and post-term.

Sepsis was defined as clinical sepsis in our hospital. Clinical diagnosis of neonatal sepsis is based on one or more signs/symptoms in at least four of the below listed groups of signs and symptoms:

a. General symptoms/signs: Ill-looking infant, infant refuses to drink, increase or decrease in body temperature, sclerema
b. Central nervous system: Lethargy, irritability, seizures
c. Cardiovascular system: Tachycardia, oedema, dehydration
d. Respiratory system: Dyspnoea, tachypnoea, cyanosis
e. Gastrointestinal system: Hepatomegaly, splenomegaly, abdominal distension
f. Haematological system: Jaundice, petechiae or bleeding, leukopenia,or the haematological score as follows: ≤ 2 very unlikely to be sepsis, 3-4 possible sepsis, ≥5 very likely to be sepsis (Table I)

Survival was defined as patients being discharged from the hospital alive or death before discharge from the hospital.

Statistical Analysis

The survival of neonates with EA was determined using a log-rank test, while the probabilities of children's survival were plotted using the Kaplan–Meier curve. The IBM SPSS Statistics version 24 (SPSS Chicago, USA) was utilised to perform all statistical analyses.

RESULTS

Baseline Characteristics

We evaluated 53 children with EA, with an overall survival rate of 18.9%. Most of them were male (52.8%), post-term (50.9%), low weight (52.8%), had thrombocytopenia (58.5%), sepsis (94.3%), pneumonia (90.6%) and had atresia with distal TEF (83%) (Table II).

Multivariate Analysis of Prognostic Factors for Survival of Children with EA

Multivariate analysis revealed that thrombocytopenia and definitive surgery were strongly associated with the survival of children with EA with a p-value of 0.014 (hazard ratio (HR)=2.67 [95% confidence interval (CI)= 1.22–5.85]) and 0.022 (HR=0.39 [95% CI=0.17–0.87]), respectively (Table IV).

DISCUSSION

Our study shows our EA children had a relatively low overall survival rate. Late diagnosis, delayed transport to tertiary care, and a lack of infrastructure are some of the causes of the high mortality rate in developing countries.[6,8,12] In contrast, the overall survival of EA patients in developed countries is 87%.[13] Some variables might have contributed to a high survival rate of children with EA, including advances in neonatal anaesthesia and intensive care and antibiotics.[12,13]

We reveal that thrombocytopenia is a strong prognostic factor for the survival of children with EA. In patients with sepsis, thrombocytopenia is caused by ingestion of platelets against direct pathogens and activation of pathogen-produced mediators, induction of apoptosis, lysis and increased clearance of phagocytes.[14] However, sepsis did not become a significant prognostic factor in our study. This finding is not compatible with previous studies that showed sepsis to be a strong prognostic factor for outcomes of children with EA.[8,15,16] Moreover, notably, thrombocytopenia can also be a surrogate marker for sepsis.

Table II: Baseline characteristics of children with EA in our institution

Characteristics	n (%)
Sex	
• Male	28 (52.8)
• Female	25 (47.2)
Weight at diagnosis (gram)	
• Normal weight (≥ 2500)	22 (41.5)
• Low weight (< 2500)	28 (52.8)
• Very low weight (< 1500)	3 (5.6)
• Extremely low weight (< 1000)	0
Gestational age	
• Preterm	15 (28.3)
• At term	11 (20.8)
• Post-term	27 (50.9)
EA type	
• Isolated EA without TEF (Gross A)	9 (17)
• EA with distal TEF (Gross C)	44 (83)
Thrombocytopenia (< 150,000/mm3)	
• Yes	31 (58.5)
• No	22 (41.5)
Pneumonia	
• Yes	48 (90.6)
• No	5 (9.4)
Sepsis	
• Yes	50 (94.3)
• No	3 (5.7)
Definitive surgery (oesophageal anastomosis)	
• Yes	17 (32.1)
• No	36 (67.9)
Outcome	
• Survived	10 (18.9)
• Died	43 (90.1)
Associated anomaly	
• VACTERL	27 (51)
• VACTERL, undescended testis	1 (1.9)
• VACTERL, Opitz G/BBB syndrome	1 (1.9)
• VACTERL, Down syndrome, clubfoot	1 (1.9)
• VACTERL, Meckel diverticulum	1 (1.9)
• VACTERL, hypospadias, undescended testis, left radial clubhand	1 (1.9)
• VACTERL, dextrocardia	2 (3.8)
• VACTERL, cholestasis	1 (1.9)
• Tracheomalacia	1 (1.9)
• No associated anomaly	14 (26.4)
• Unknown	3 (5.7)

EA: Oesophageal atresia; TEF: Tracheoesophageal atresia

Table IV: Multivariate analysis of survival of children with EA in our institution

Variables	HR (95% CI)	p-value
Sex	0.82 (0.42–1.63)	0.578
EA type	0.69 (0.23–2.02)	0.496
Thrombocytopenia	2.67 (1.22–5.85)	0.014*
Pneumonia	3.67 (0.84–16.04)	0.084
Sepsis	0.80 (0.12–5.41)	0.817
Definitive treatment	0.39 (0.17–0.87)	0.022*

*, $p < 0.05$; CI: Confidence interval; HR, hazard ratio; EA, oesophageal atresia

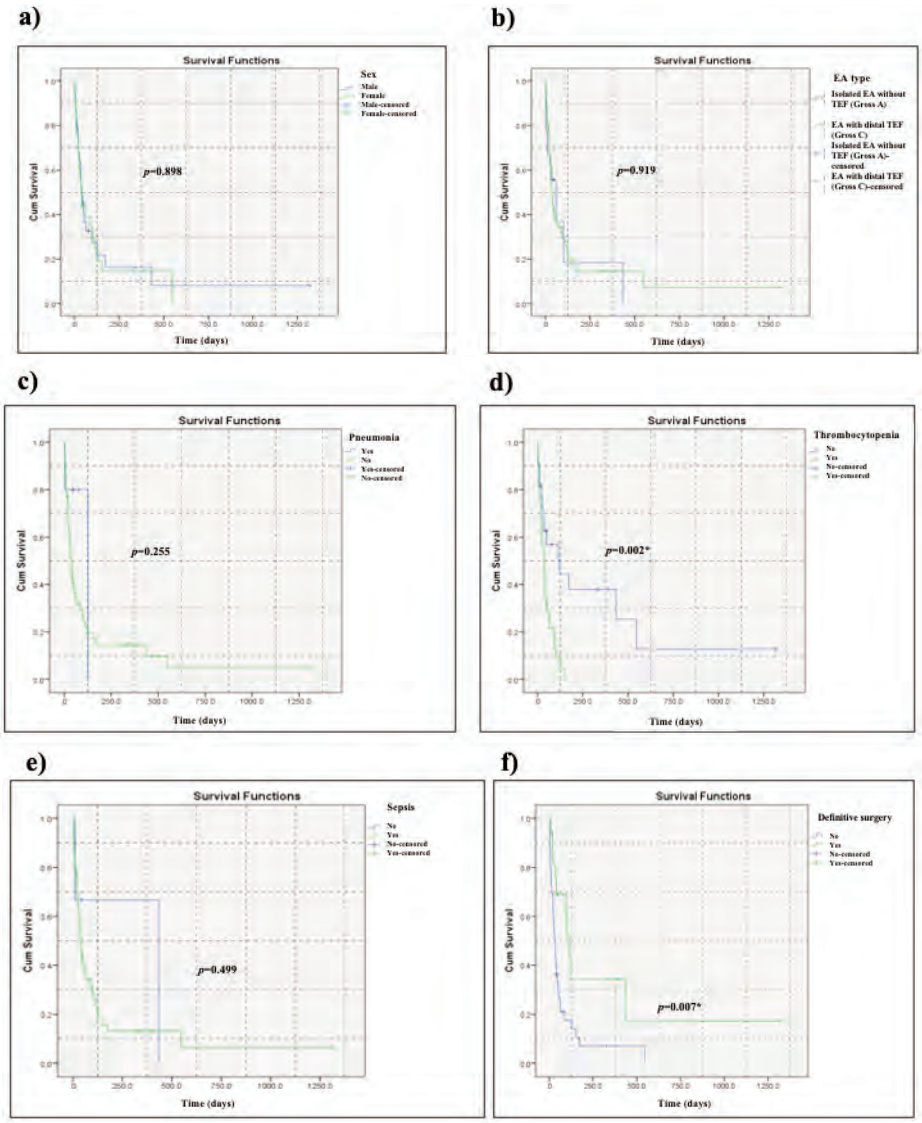

Fig. 1: Kaplan-Meier analysis for the association between prognostic factors: a) sex, b) EA type, c) pneumonia, d) thrombocytopenia, e) sepsis, f) definitive surgery, and EA patients' survival. A log-rank test showed that thrombocytopenia and definitive surgery had a significant association with the survival of EA patients, with a p-value of 0.002 and 0.007, respectively. Whereas, sex, EA type, pneumonia and sepsis were not (p = 0.898, 0.919, 0.255 and 0.499, respectively).

We fail to show pneumonia as a significant prognostic factor for the survival rate of children with EA which is different from previous study.[17] When air passes through the fistula, it causes stomach distension and subsequent reflux of gastric contents through the same TEF, causing aspiration pneumonia.[18] Airway abnormalities such as tracheomalacia, tracheobronchial malformations and lung hypoplasia, according to a previous study, contribute to recurrent respiratory exacerbations by impairing mucociliary transport.[19]

Interestingly, our finding shows that children with EA who underwent definitive surgery have a higher survival rate than subjects who did not undergo oesophageal anastomosis. However, previous reports revealed that definitive surgery did not affect the overall survival of EA patients.[6]

Our study provides new evidence of the association between the survival rate of EA patients with several prognostic factors, including thrombocytopenia and definitive surgery, from a particular developing country in Southeast Asia, Indonesia.

Several limitations have been noted in our study, including a single centre study, which would lead to an inadequate sample size. We also have difficulties analysing the long-term consequences of EA because of its retrospective design. In addition, we associated the outcomes of EA patients with prognostic factors according to overall means without considering other variables that might impact this association, including surgery experiences, late diagnosis and delayed transport to tertiary care.

CONCLUSION

Our study shows that thrombocytopenia might decrease the survival of children with EA, while definitive surgery might be beneficial for the survival of children with EA. It implies that definitive surgery should be performed as early as necessary to prevent further morbidity and mortality. Our study comprehensively provides the survival of children with EA and links it to prognostic variables in a particular developing country. It serves as a potential research project that can be applied to the clinical setting to help clinicians manage EA better.

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This study was approved by the Institutional Review Board of the Faculty of Medicine, Universitas Gadjah Mada/Dr. Sardjito Hospital, Yogyakarta, Indonesia (KE/FK/0963/EC/2020). Written informed consent was obtained from all parents who participated in this study. The research has been performed following the Declaration of Helsinki. Some results for the manuscript are from Andi Lestiono's thesis.

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Characteristics and management of conjoined twins: A single-centre retrospective descriptive study

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ABSTRACT

Introduction: Conjoined twins (CT) is a rare congenital disorder characterised by the presence of malformations associated with secondary abnormal conjoined organ changes and abnormal hemodynamic superimposed effects about 1 in every 200 identical twin pregnancies, between 1 in 50,000 to 1 in 100,000 live births. The aim of this study is to describe the characteristics of conjoined twins.

Materials and methods: This was a retrospective descriptive study. All medical records of conjoined twins who were admitted to Hasan Sadikin Bandung General Hospital from January 1st, 2015, to June 30th, 2023, were reviewed for gender, conjoined type, birth order, risk factor and treatment.

Results: Of the 28 conjoined twins, 21 were girls (75%), and 7 were boys (25%); 19 (67,85%) were of the thoracoomphalopagus type; 11 (39,28%) were born as first children; 18 (64,28%) were born at 37 weeks of gestational age; and 22 twins' (78,57%) parents were aged between 21 and 35 years. None of the mothers had used medication, 13 (46,42%) took folic acid on occasion, five (17,85%) used traditional herbs, nine (32,14%) smoked and none drank alcohol. Parents who live in industrial areas were 18 (64.28%). There was no history of conjoined twins in previous pregnancies or deliveries or in the parent's family. Liver separation had been done in four (14.28%). Emergency separation in one twin. Nine (21.42%) patients died before surgery due to a worsening condition.

Conclusion: The conjoined twins were more common in girls, predominantly of the thoracoomphalopagus type. Risk factors that were commonly found were the first child, a gestational age of less than 37 weeks, and living in an industrial area.

KEYWORDS:
Conjoined twins, gestational age, medication, industrial area

INTRODUCTION

Conjoined twins (CT) are one of the rarest types of monochorionic monoamniotic twins. The incidence ranges from one out of every 200 identical twin pregnancies to almost always being identical, between one in 50,000 to one in 100,000 live births.[1,2] Some studies report prevalence as high as one in 2,800 live births in India,[3] and as low as one in 200,000 live births in the USA.[4] However, prevalence increases of 3.27: 100,000 live births[5] and 2.85: 100,000 live births[6] were reported in two studies in the Chinese population of surveillance programs at different times. A higher incidence is found in Africa, about 1: 14,000, and in Asia, 1: 25,000.[7]

CT are malformations associated with secondary changes in abnormal conjoined organs and abnormal hemodynamically superimposed effects.[8] The mechanism of the defect is due to changes in normal development processes, in which a pair of monozygotic twins (MZ) do not separate completely from one another and continue normal embryological development. MZ twins start with the separation and division of a single early embryo.[9-11] The mechanism of CT is still unclear. There are conflicting theories explaining the sequence of events in CT. CT are derived from a single fertilised egg. There are two theories to explain this phenomenon: 1) Fusion theory (more accepted): when a single fertilised egg is divided into two embryos. The phenomenon occurs between 13 and 15 days after fertilisation, resulting in failure to complete division. 2) The fission theory: when there is union of two embryos originally separated about 12 days after fertilisation. The theory supporting the 'fusion' process cannot explain the parapagus type, but it can explain the other type by the fusion of two separate embryos.[12-16] The formation of the parapagus can be explained by a single notochord bifurcation.[17] No theory of embryonic vertebral fission at any stage of development, from various planes and from various directions, can explain the selection of fusion sites, the details of fusion, or the delimitation of the specific area where twins are found joined.[13] On the other hand, proponents of fission theory claim that CT is the result of imperfect separation of the embryonic axis.[8,9,18-22] Except for parasitic twins, all CTs are symmetrical, and 'equal parts always join equal parts.'[9] The same authors state, 'If fusion, rather than fission, occurs in all cases of conjoined twins, the incidence of similar appearance is similar in all monoamniotic twins, whether conjoined or not,' and 'if the incidence of similar appearance is higher in CT than in separated twins, the fusion hypothesis is incorrect.' There are no records in the literature regarding the familial aggregation of CT or its preferential association with other unrelated anomalies.[23] Recently, it was found that low-dose radiation exposure triggers the incidence of twinning and the prevalence of CT.[24]

About 75% of conjoined twin pairs are female, with a female-to-male ratio of 3:1. Of the total, about 40% are stillborn, and 60% are live births; only about 25% live long enough to be

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candidates for surgery.[7] CT are classified based on the most common location of connection: thorax (thoracopagus), abdomen (omphalopagus), sacrum (pygopagus), pelvis (ischiopagus), skull (cephalopagus) and back (rachipagus). Based on the aspect of the embryonic disc, the most common type is the thoracopagus (19%).[25] Omphalopagus is the least common, with an incidence of 0.5%.[26] The overall survival rate of CT is around 25%.[27]

In recent studies, the diagnosis can be made in the first trimester, and because the family has already opted for the termination of pregnancy, further diagnostic intervention is not necessary. However, in developing countries, the lack of adequate maternal care facilities causes late diagnosis. Because this situation poses a great risk, early diagnosis and treatment during delivery are very important. Surgical separation of CT can vary from minimal risk to very complex, depending on the point of attachment and the internal parts that are shared.[1,2] This study wants to describe the characteristics of conjoined twins.

MATERIALS AND METHODS

This was a retrospective descriptive study at Hasan Sadikin Bandung General Hospital. From January 1st, 2015, to June 30th, 2023, 28 patients' data were collected on gender, conjoined type, birth order, gestational age, parent age, history of medication, traditional herbal, cigarette and alcohol consumption, residence and treatment.

RESULTS

There were few case series as example to compare characteristic in conjoined twins found at Hasan Sadikin Bandung General Hospital.

The patient was consulted from the paediatric department with the following diagnosis: conjoined twin parapagus + dd/Ebstein anomaly + pulmonary stenosis + single ventricle + hypoplasia distal phalanx digiti III-V manus dextra + CTEV (congenital talipes equino varus) + suspect CCAM (congenital cystic adenomatoid malformations). Attached body parts were the abdomen with two limbs, one umbilicus, one external genitalia, and one anus. Babies are born at a gestational age of 38 to 39 weeks.

There were one penis and one scrotum, one anus, two inferior extremities and the right lower extremity looked pale. On radiography, two vertebrae and one pelvis were found; on ultrasound, two livers and two separate bladders were found; on echocardiography, baby A with PDA and baby B with DORV (double outlet right ventricle), inlet VSD, CA-VSD, mild coarctation of the aorta, valvular PS, PDA: left to shunt right, and PLSVC (persistent left superior vena cava). Intraoperatively, each baby was found with 1 kidney facing into the pelvic area, with each ureter connected to one bladder, and two testicles in the pelvic area with normal shape, half the normal size, looked vital. The sacrococcygeal fusion was connected by fibrous tissue. Liver fusion (2.5 cm long, 1.5 cm thick) was separated with a harmonic scalper; ileal fusion 20 cm proximal to the Bauhini valve, sutured with 3.0 silk, with a single terminal ileum, caecum, ascending colon, and transverse colon. Duplicated colon in infant A, from splenic flexure to rectum, with fenestrated shared walls Meckel's diverticulum was found in baby A, 12 cm proximal to the Bauhini valve. Ileal fusion separated in infant B, proximal to ileal fusion. Left congenital diaphragmatic hernia was found in baby B (the organs that entered were the stomach, small intestine, left lobe of the liver and spleen)—a defect of about half the diaphragm. The intestines were separated. With an anterior incision approach, the pubic symphysis was separated, and the sacrum was separated into the right superior and inferior pubic rami. Postoperative findings were infant A with a complete gastrointestinal tract, from stomach to rectoanal, with duplication of descending colon to rectum, liver and spleen. Baby A was found with one left kidney, one left ureter connected to the bladder and urethra, one left testicle, one pelvis and one left extremity. The abdomen was closed in the same way as the first, and the part that had not been closed was given a prosthetic mesh to close the defect. Cutis and subcutis dissected the stretched part. The patient died 2 days postoperatively due to septic shock, hypoalbuminemia and suspected acute left lower extremity thrombosis.

One case of female thoracoomphalopagus was performed for emergency separation on the 4th day of life because baby 1 was declared dead. The patient was born as a preterm infant (31 to 32 weeks) and was not crying immediately. A liver fusion was found, each of which has a biliary vascular tree. In the intestines, no fusion was found, each having its own gastrointestinal system and its own urogenital system. A cross-vessel liver was found in the upper 1/3 of the fusion, with a diameter of 0.5 cm. Fusion was found in the proximal arch of the xiphoid process of the ribs. There was pericardial fusion at the apex ± 0.5 cm and intravesical pressure of 12.6 mmHg.

There were 3-year-old male conjoined twin ischiopagus with three inferior extremities, one bladder, and a shared intestine with one rectum, suggesting left kidney hypoplasia in conjoined twin 1 and left kidney agenesis in conjoined twin 2. The patient was born at term (40 weeks) and was crying immediately. In conjoined twin 1, forming the hepatogastric trunk (coeliac trunk variant type III, based on Uflacker's classification). Normal structures of the splenic parenchyma and splenic arteries are not visualised. The left renal artery appears smaller than the right renal artery. Patients underwent disarticulation of the supernumerary limbs, pelvic wedge osteotomy, ORIF P/S, tension band wiring and defect closure with a local advancement flap. Incisions in the proximal supernumerary limb were performed layer by layer; fat exposure, fascia and limb disarticulation were done. Wedge osteotomy at the pelvic bone, then pelvic reduction and approximation with SS wire. Drilling and setting recon plate surrel locking 6 mm with four cancellous screws, two screws installed, and SS wire tension band wiring There was a peritoneal defect measuring 6 cm in diameter with a small intestine exposed. Peritoneal defect closure was performed with chromic 4-0. At the regiolateral abdomen, there was an open wound post-disarticulation by an orthopaedic surgeon, 12 × 5 × 8 cm in size, with a muscle and fascia base.

Table I: Characteristics of patients with conjoined twins

Demography characteristic	Frequency (n = 28)
Gender	
• Girl	21 (75%)
• Boy	7 (25%)
Conjoined twins type	
• Thoracoomphalopagus	19 (67,85%)
• Abdominopygopagus	2 (7,14%)
• Ischiopagus	2 (7,14%)
• Cephalopagus	4 (14,28%)
• Parapagus	1 (3,57%)
• Rachipagus	0 (0%)
Birth order	
• First	11 (39,28%)
• Second	8 (28,57%)
• Third	9 (32,14%)
Gestational age	
• < 37 weeks	18 (64,28%)
• ≥ 37 weeks	10 (35,71%)
Parent age	
• < 21 years	6 (21,42%)
• 21-35 years	21 (78,57%)
• >35 years	0 (0%)
History of medication consumption	
• Analgesic and stomach ulcers medicine	4 (14,28%)
• None	24 (85,71%)
History of folic acid consumption	
• Always	8 (28,57%)
• Sometimes	13 (46,42%)
• Never	7 (25%)
History of traditional herbal consumption	
• Ever	5 (17,85%)
• Never	23 (82,14%)
Habitual smoking	
• Mother	0 (0%)
• Father	9 (32,14%)
Alcohol consumption	
• Positive	0 (0%)
• Negative	28 (100%)
Living in the industrial area	
• Yes	18 (64,28%)
• No	10 (35,71%)
History of childbirth with previous conjoined twins	
• Positive	0 (0%)
• Negative	28 (100%)
History of conjoined twins occurrence in family	
• Mother	0 (0%)
• Father	0 (0%)
Treatment	
• Rudimentary limb ablation	2 (7,14%)
• Bladder reconstruction	1 (3,57%)
• Liver separation	4 (14,28%)
• Abdominal wall reconstruction	4 (14,28%)
• Sternal and pericardial separation	3 (10,71%)
• Chest wall reconstruction	3 (10,71%)
• Disarticulation of the supernumerary limbs	1 (3,57%)
• None	20 (71,42%)
Inoperable	6 (21,42%)
Plan for elective surgery	5 (17,85%)
Death	9 (31,14%)

Data are presented as n (%)

Fig. 1: (Left) Two-months-old twin with thoracoomphalopagus, fusion of the chest and upper part of the abdomen with suspect congenital heart disease (right) Two-days-old cephalopagus (temporal bone fusion)

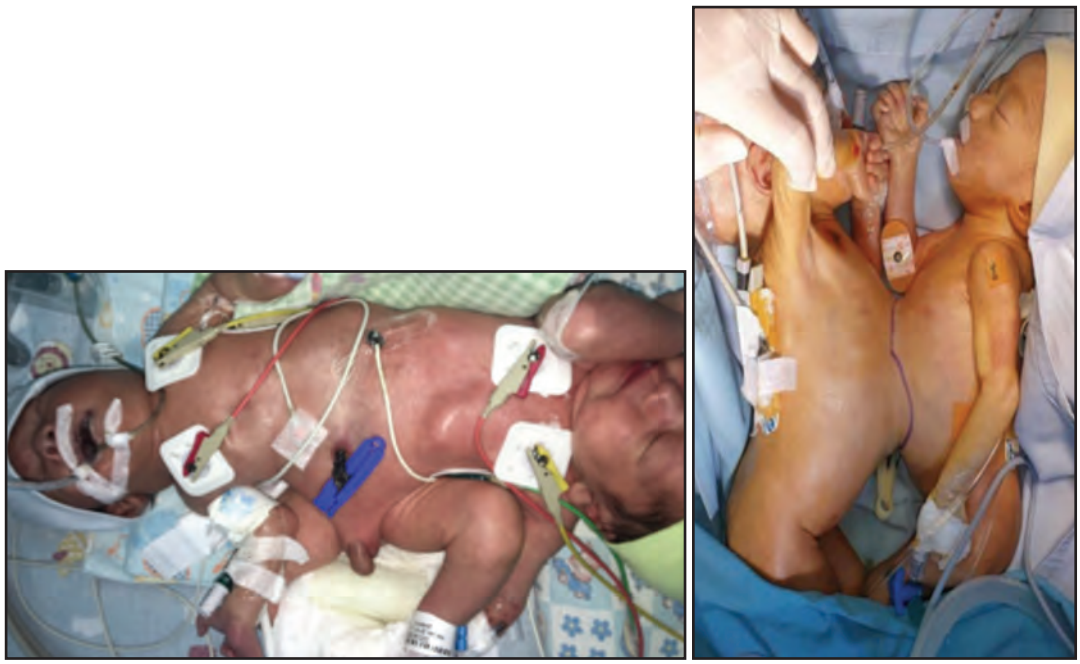

Fig. 2: (Left) One-day-old conjoined twin parapagus that the abdomen was attached with two limbs, one umbilicus, one external genitalia and one anus (right) Four-days-old thoracoomphalopagus with liver, proximal arch of the xiphoid process of the ribs, and pericardial fusion. Baby 1 died

There were six inoperable CT because of shared internal organ such as intestine and liver, shared external organ such as leg, penile, anus or inseparable ventricle and atrium of heart. Meanwhile nine twins were death before surgery due to worsening condition of asphyxia, pneumonia, sepsis, congenital heart disease such as tetralogy of Fallot, tricuspid atresia, ventricular septal defect etc.

DISCUSSION

The occurrence of CT is influenced by many factors that are not clearly known. The risk factors observed in this study were gender, birth order, gestational age, parent age, consumption of medication, folic acid, cigarettes and alcohol, living in an industrial area, history of CT in previous pregnancies or deliveries, and history of CT occurrence in family. Of 28 conjoined twins, 21 girls (75%) and seven boys (25%), according to one study, showed a 1.5–2.5 female sex predominance over male sex.[2]

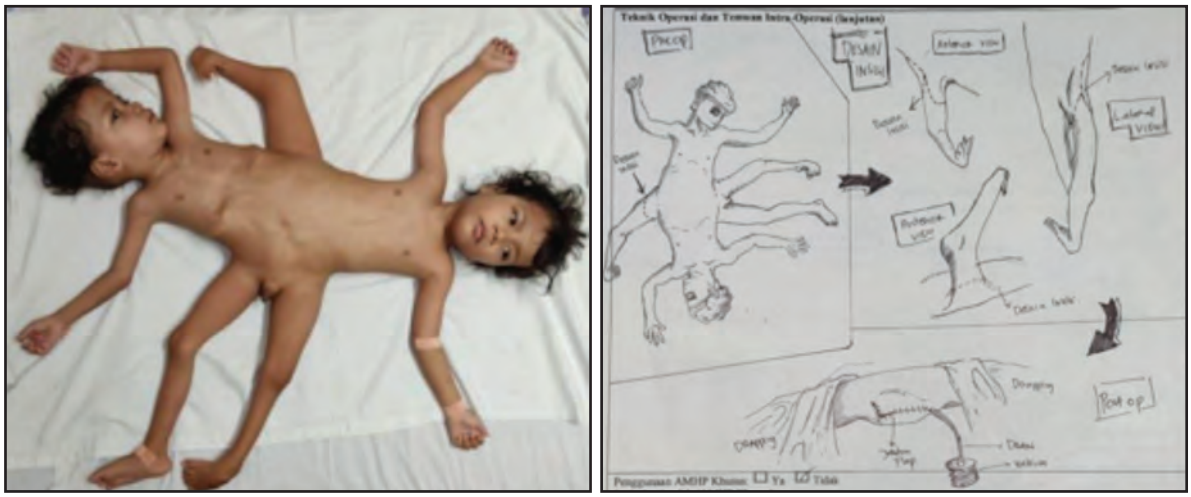

Fig. 3: (Left) 3-year- old ischiopagus with three inferior extremities, one bladder, shared intestine, and one rectum (right) incision design of surgery

A total of 19 twins (67,85%) were thoracoomphalopagus types, but based on the aspect of the embryonic disc, the most common type is thoracopagus (19%).[25] Omphalopagus is the least common, with an incidence of 0.5%.[26] 11 (39,28%) were born as first children; 18 (64.28%) were born at 37 weeks of gestational age; and 22 twins' (78,57%) parents aged between 21 and 35 years, the same as demographic characteristics such as mother's age, reproductive data, including gestational age, birth weight, and environmental factors such as exposure to and disease in the mother during pregnancy.[28]

In our series, risk factors such as consumption of medication, folic acid, traditional herbal and cigarettes were found in a small number (medication 0%, folic acid 13 (46,42%), traditional herbal 5 (17,85%) and cigarette 9 (32,14%)). No alcohol consumption was found. 18 (64.28%) CT were living in industrial areas but still required further study considering the limited number of samples for statistical tests. There was no history of CT in previous pregnancies or deliveries or in the parent's family. Generally, multiple factors, such as alcohol consumption, medication use, radiation or chemical exposure, and lack of folic acid supplementation during pregnancy, as well as family history, are reported risk factors for the development of congenital anomalies. Specific to conjoined twins, the exact aetiology has not been discovered yet.[29]

CONCLUSION

The incidence of CT was more common in girls, predominantly the thoracoomphalopagus type, with gestational age in first-born children at less than 37 weeks' gestation who were living in the industrial area. There was no history of consumption of medication, especially teratogenic, in this study. In the future, hopefully further research can be carried out regarding the incidence of CT being influenced by other risk factors and the management of conjoined twins.

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Chlorogenic acid ameliorates muscle wasting by upregulating mRNA expressions of calcineurin and PGC-1α in diabetic rat model

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ABSTRACT

Introduction: Muscle health in diabetes mellitus (DM) is often neglected, which leads to muscle wasting. Increased reactive oxygen species in DM could decrease antioxidant enzymes such as superoxide dismutase-1 (SOD-1) and -2 (SOD-2) and inhibit calcineurin (CN) and PGC-1α signalling pathways. Chlorogenic acid (CGA) is known as a potent antioxidant and activators of CN and PGC-1α. This study aimed to determine the effect of CGA on mRNA expressions of SOD-1, SOD-2, CN and PGC-1α in inhibiting the progression of DM to muscle wasting.

Materials and Methods: This study was conducted at Department of Anatomy, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada starting on July 20th, 2020. A total of 24 male Wistar rats were randomly divided into six groups (four rats per group), i.e., control, DM 1.5 months (DM1.5), and DM 2 months (DM2); and DM groups treated with CGA in three different doses, namely CGA1 (12.5 mg/kg BW), CGA2 (25 mg/kg BW), and CGA3 (50 mg/kg BW). Control group was only injected with normal saline, while diabetic model was induced by intraperitoneal injection of streptozotocin. Blood glucose levels were measured twice (one week after diabetic induction and before termination). The soleus muscle tissue was harvested to analyse the mRNA expressions of SOD-1, SOD-2, CN and PGC-1α using RT-PCR. In addition, the tissue samples were stained with immunohistochemistry for CN and haematoxylin-eosin (HE) for morphologic analysis under light microscopy.

Results: The mRNA expressions of SOD-1 and SOD-2 in the CGA1 group were relatively higher compared to the DM2 groups. The mRNA expression of CN in the CGA1 group was significantly higher compared to the DM2 group (p = 0.008). The mRNA expression of PGC-1α in the CGA1 group was significantly higher compared to the DM2 group (p = 0.025). Immunohistochemical staining showed that CN-immunopositive expression in the CGA1 group was more evident compared to the other groups. Haematoxylin-eosin staining showed that muscle tissue morphology in the CGA1 group was similar to that in the control group.

Conclusion: Chlorogenic acid at a dose of 12.5 mg/kg BW shows lower blood glucose level, good skeletal muscle tissue morphology and higher mRNA expressions of SOD-1, SOD-2, CN and PGC-1α compared to the DM groups.

KEYWORDS:
Muscle wasting, calcineurin, PGC-1α, SOD-1, SOD-2

INTRODUCTION

Diabetes mellitus (DM) poses a significant global public health concern, impacting an estimated 422 million adults worldwide in 2014.[1] It contributed to 1.5 million deaths globally in 2012.[2] While in Indonesia, DM ranks second as the leading cause of death, accounting for 6% of total deaths in the population.[3] DM is characterised by high oxidative stress resulting from chronic hyperglycaemia, which can affect protein metabolism.[4] An increase in antioxidant enzymes, such as superoxide dismutase-1 (SOD-1) and superoxide dismutase-2 (SOD-2), will reduce reactive oxygen species (ROS), preventing the activation of signalling pathways that could induce protein degradation.[5]

DM, associated with abnormal muscle protein metabolism, can result in decreased muscle mass and, in some cases, affect activities of daily living, leading to decreased productivity and quality of life.[6] Muscle proteins are continuously synthesised and degraded every day, typically balancing to maintain muscle mass. However, in DM, factors such as decreased synthesis or increased breakdown can result in an imbalance leading to muscle wasting.[7] Muscle wasting is a condition characterised by the loss of muscle mass, which occurs due to an imbalance between protein synthesis and degradation.[8] There are several triggers for muscle wasting, including decreased calcineurin (CN) activity, especially in cases of chronic DM. Calcineurin is involved in several adaptive responses that induce growth and regeneration of muscle fibres.[9] Decreased CN activity, as a result of suppression by conditions that trigger atrophy, leads to the reduction of various mediators that serve as key components of protein degradation in muscle.[10] Peroxisome proliferator-activated receptor gamma coactivator-1 alpha

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(PGC-1α) is associated with the regulation of skeletal muscle protein turnover.[11] Decreased CN activity will downregulate PGC-1α, resulting in decreased muscle functional capacity and muscle mass, which often accompanies DM.[12]

Chlorogenic acid (CGA) is a polyphenol compound in coffee with the highest antioxidant content.[13] Chlorogenic acid can stimulate glucose transport in skeletal muscle and offers numerous benefits in the management of DM and its complications.[14] Changes in protein metabolism due to oxidative injury and their impact on muscle mass represents one of the most challenging and poorly understood aspects of DM management. The results of this study are expected to serve as a reference for understanding the progression of DM and the potential use of CGA as an innovative therapy to maintain optimal physical capacity and functional abilities in DM patients. Therefore, it is necessary to conduct further studies to investigate the effect of CGA on muscle wasting in diabetic rats, focusing on the mRNA expressions of SOD-1, SOD-2, CN and PGC-1α.

MATERIALS AND METHODS

Design

This study was a quasi-experimental study with a post-test-only controlled group design. This study was conducted at the Department of Anatomy, Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada (FM-PHN UGM) starting on July 20th, 2020. This study has received approval from the Medical and Health Research Ethics Committee FM-PHN UGM/RSUP Dr. Sardjito, with the reference number KE/FK/1086/EC/2020.

Animal Model of Diabetes Mellitus

A total of 24 male Wistar rats aged 2 months old with a body weight (BW) ranging from 150 to 200 grams were obtained from the Muhammadiyah University of Yogyakarta. The animals were housed in cages (2 rats per cage) with light-dark cycle of 12 hours, controlled temperature (21 ± 2°C) and humidity (50 ± 5%), and given free access to food and water. Healthy rats were included in this study, while sick rats, characterised by reduced activity, decreased body weight and hair loss, were excluded from the study. The rats (n = 24) were divided into six groups: control, DM1.5 (DM for 1.5 months), DM2 (DM for 2 months), and three treatment groups with different dosages of CGA. The control group was injected with normal saline. Diabetic induction model was prepared by intraperitoneal injection of streptozotocin (STZ) at a dose of 60 mg/kg, dissolved in 0.1 M citric acid with a pH of 4.5. Blood samples of fasting rats were withdrawn from the tail vein at one week after diabetic induction. Diabetes condition was defined as blood glucose levels greater or equal to 250 mg/dL using a portable glucometer. Blood glucose levels were also measured before termination (Figure 1).

Chlorogenic Acid Administration

CGA was dissolved using PBS and administered via intraperitoneal injection at a total volume of 1 mL/kg BW. Three doses of CGA were used: 12.5 mg/kg BW (CGA1), 25 mg/kg BW (CGA2) and 50 mg/kg BW (CGA3). The CGA groups received treatment for 14 consecutive days (Figure 1).

Termination and Sample Harvesting

Termination was carried out on day 60 in accordance with the AVMA guidelines for the euthanasia of animals (2020 edition). Experimental animals were euthanised using ketamine at a dose of 100 mg/kg BW, administered intraperitoneally. The soleus muscle tissue was then quickly harvested and stored in a 1.5 mL tube filled with RNA preservation solution at -20°C.

Reverse Transcription PCR

Soleus muscle tissue was used for RNA extraction and the extracted RNA was then employed for cDNA synthesis. The synthesised cDNA was used in reverse transcription PCR (RT-PCR) reactions and then followed by electrophoresis. The mRNA expression levels for SOD-1 (F: GCGGTGAACCAGTTGTGGTG; R: AGCCACATTGCCCAGGTCTC), SOD-2 (F: ATGTTGTGTCGGGCGGCGTGCAGC; R: GCGCCTCGTGGTACTTCTCCTCGGTG), CN (F: AGTAACTTTCGAGCCAGCCC; R: CAACGCGACACTTTCTTCCAG), and PGC-1α (F: TCAGCGGTCTTAGCACTCA; R: TCTCTGTGGGTTTGGTGTGA) were determined through densitometric analysis using ImageJ software (NIH). The β-actin was used as a housekeeping gene (F: GCAGATGTGGATCAGCAAGC; R: GGTGTAAAACGCAGCTCAGTAA).

Immunohistochemical Staining

A formalin-fixed paraffin-embedded tissue sample was simultaneously stained using immunohistochemical (IHC) technique for CN. The sections were incubated with anti-CN antibody (rabbit polyclonal, Abcam; 1:300 dilution) and the Mouse/Rabbit Probe HRP Labelling Kit with DAB Brown (BioTnA) as the chromogen. Observation of the IHC-stained sections was carried out under a light microscope at a magnification of 400x, covering the entire field of view of the muscle tissue. The assessment was based on the observation of brown coloration in the longitudinal section of the soleus muscle tissue.

Haematoxylin-Eosin Staining

A formalin-fixed paraffin-embedded tissue sample was simultaneously stained using haematoxylin-eosin (HE) for each group. HE staining was carried out to visualise the muscle tissue morphology and the HE-stained sections were then observed under a light microscope at a magnification of 100x.

Data Analysis

The data were assessed using the Shapiro-Wilk test to determine the data distribution. One-way ANOVA test was employed and followed by post-hoc least significant difference (LSD) test for normally distributed data. While Kruskal-Wallis test was carried out and followed by post-hoc Mann-Whitney test for non-normally distributed data. A significance level of $p < 0.05$ was used as the criteria for statistical significance in all analyses.

RESULTS

Blood Glucose Level

The measurements of blood glucose level were conducted twice in each group: in the first week following diabetic induction and before termination (Figure 2). The results showed that the blood glucose level in the first week following diabetic induction in the control group (101 ± 11.9 mg/dL) was significantly lower compared to both the DM and treatment groups, namely, DM1.5 (342. 5 ± 53 mg/dL; p = 0.021), DM2 (266 ± 41.1 mg/dL; p = 0.021), CGA1 (389.5 ± 213.2 mg/dL; p = 0.021), CGA2 (459.25 ± 156.3 mg/dL; p = 0.021), and CGA3 (507.5 ± 72.7 mg/dL; p = 0.020). Furthermore, the DM2 group exhibited a significant higher blood glucose level before termination (594.5 ± 115.4 mg/dL) compared to the CGA1 (136.75 ± 4.57 mg/dL; p = 0.021) and CGA2 groups (238 ± 148.9 mg/dL; p = 0.043). Moreover, the blood glucose levels in the CGA1 and CGA2 groups were significantly lower compared to the CGA3 group. These results indicated that blood glucose levels were higher in the DM rats and CGA administration had a noticeable effect in reducing blood glucose levels.

SOD-1 and SOD-2 mRNA Expressions

The SOD-1 densitometric analysis data were initially assessed using the Shapiro-Wilk test for normality, which indicated that all groups had a normal distribution data ($p \geq 0.05$). Subsequently, a one-way ANOVA test was carried out followed by post-hoc LSD analysis, suggesting no significant difference between all groups (p = 0.450). However, mRNA expression of SOD-1 in CGA1 group was higher compared to the DM2 group. These findings indicated that CGA might have an effect on increasing mRNA expression of SOD-1 (Figure 3).

The mRNA expression of SOD-2 in the CGA1 group were significantly higher compared to the CGA3 group (p = 0.039). Moreover, the mRNA expression of SOD-2 in CGA1 group was relatively higher compared to the DM2 group. These results suggested that mRNA expression of SOD-2 was decreased in the DM groups and CGA appeared to have an effect on increasing mRNA expression of SOD-2 (Figure 3).

Calcineurin and PGC-1α mRNA Expressions

The statistical test results showed that mRNA expressions of CN in the DM2 (p = 0.016) and CGA2 (p = 0.029) groups were significantly lower compared to the control group. On the contrary, mRNA expression of CN in the CGA1 group was significantly higher compared to the DM2 group (p = 0.008). These results indicated that mRNA expression of CN was decreased in the DM rats and CGA appeared to have an effect on increasing mRNA expression of CN (Figure 3).

The statistical test results showed that mRNA expressions of PGC-1α in the DM1.5 (p = 0.028) and DM2 (p = 0.002) groups were significantly lower compared to the control group. In contrast, mRNA expression of PGC-1α in the CGA1 group was significantly higher compared to the DM2 group (p = 0.025). These results indicated that mRNA expression of PGC-1α was decreased in DM rats and CGA appeared to have an effect on increasing mRNA expression of PGC-1α (Figure 3).

Calcineurin Expression in Soleus Muscle Tissue

Immunohistochemical staining (Figure 4) of soleus muscle tissue sections revealed that CN-immunopositive expression in the DM groups were less abundant compared to the control group. In contrast, CN-immunopositive expression in the control and CGA1 groups were more abundant compared to the other groups.

Histopathological Examination of Skeletal Muscle Tissue

The histopathological examination of muscle tissue in the control group depicted a typical skeletal muscle morphology under normal condition (Figure 5). In the histopathological images of HE-stained longitudinal sections, differences in muscle structure were observed in the DM groups compared to the control group. Specifically, the muscle structure in the DM groups exhibited irregular myofibers with stacked and irregular myonuclei compared to the control group. In contrast, the CGA1 group displayed muscle structure similar to that of the control group, in which myofibers and myonuclei appeared more regular compared to the DM groups. Furthermore, the CGA1 group exhibited improved muscle morphology compared to both CGA2 and CGA3 groups.

DISCUSSION

In this study, the CGA administration is observed to enhance the skeletal muscle performance and inhibit the progression of muscle wasting in terms of reduced blood glucose level, transcriptomic signalling pathways, immunohistochemical parameters and histopathological muscle structure. Notably, in the CGA1 group with a dose of 12.5 mg/kg BW, improvements in skeletal muscle structure are more evident in its histopathological features, along with reduced blood glucose level compared to other treatment groups. Furthermore, the CGA1 group exhibits relatively higher mRNA expression of SOD-1 and SOD-2, serving as markers of antioxidant enzymes. These increases in mRNA expressions of antioxidant enzymes are in line with the potential to reduce oxidative stress. Additionally, CN and PGC-1α mRNA expressions are also higher in the CGA1 group, suggesting their roles in promoting protein synthesis and mitochondrial biogenesis to prevent muscle wasting.

Skeletal muscle consists of multinucleated myofibers (or myotubes) and satellite cells, all enclosed within the sarcolemma. A single myofiber contains the nucleus, myofilaments, sarcoplasmic reticulum and mitochondria, which performs to supply energy for movement.[11] In this study, the histopathological examination of HE-stained skeletal muscle tissue shows changes in the morphology of skeletal muscle cells in the DM groups, which are different from the morphology of skeletal muscle cells in the control group. These morphological changes are characterised by damage to the boundaries between myofibers and irregular myonuclei in the DM groups. Our result is supported by previous research that shows that skeletal muscles in DM undergo changes in its structural features, including myofibril damage, absence of Z-lines, irregular mitochondria, increased fat content, folded sarcolemma, irregular myonuclei and hyperchromatism.[12]

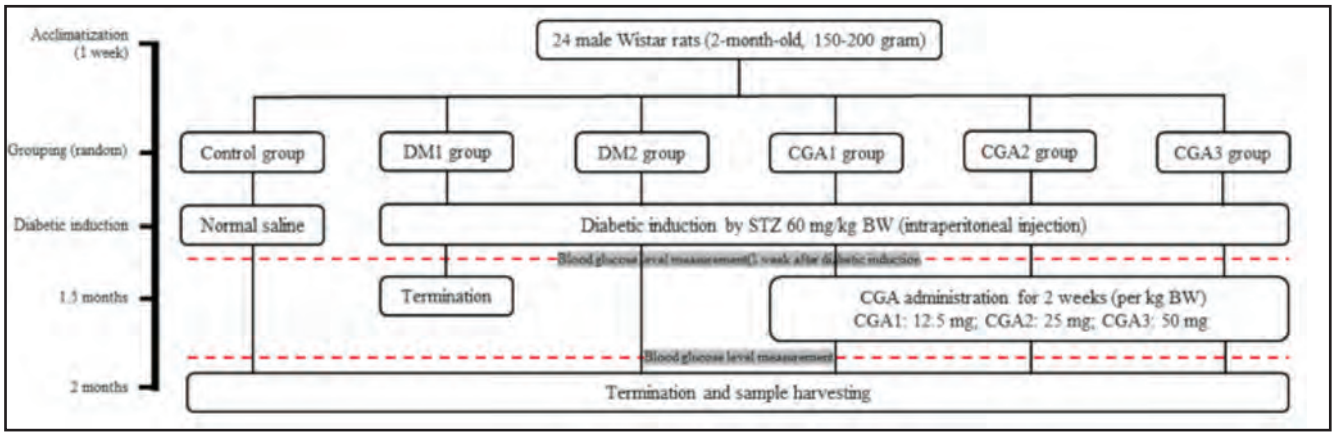

Fig. 1: Schematic diagram of research design

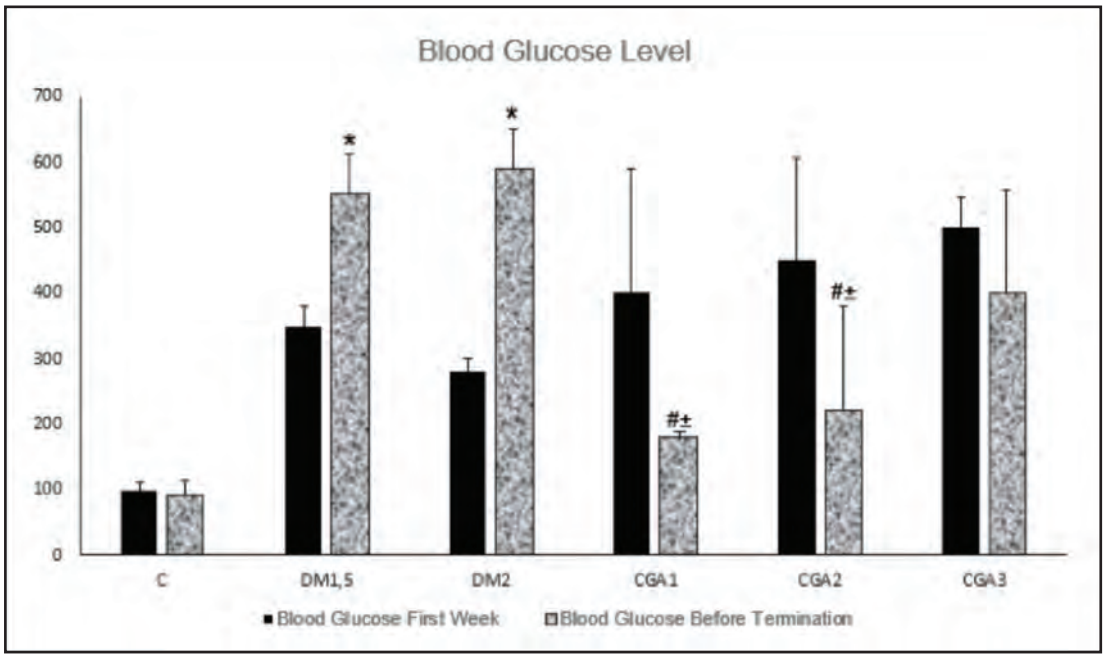

Fig. 2: Blood glucose level in the first week following diabetic induction and before termination (mean ± SD mg/dL) in the control, DM and CGA groups. *: significantly different from control group; #: significantly different from DM2 group; ±: significantly different from CGA3 group

Increased systemic ROS production in cases of DM can increase oxidative stress and lead to changes in peripheral tissues such as skeletal muscle, along with an increase level of proinflammatory transcription factors such as nuclear factor kappa B (NF-κB). The NF-κB regulates specific UPS genes that can trigger protein degradation and results in muscle wasting.[4] In the CGA1 group, the histological morphology is similar to that of the control group. The morphological changes observed in the CGA1 group are attributed to the administration of CGA, which can increase the expression of antioxidant enzymes. These enzymes play roles to stabilise redox conditions and reduce ROS levels, thereby preventing pro-inflammatory reactions and the potential damage to muscle structure and protein degradation. Our study result is supported by previous study indicating that CGA, as an antioxidant, could reduce free radicals, enhance antioxidant enzymes and inhibit oxidation reactions.[13] An increase in antioxidant enzymes such as SOD-1 and SOD-2 can effectively reduce ROS levels, preventing the activation of the NF-κB signalling pathway, which, in turn, can prevent the induction of MurF-1 expression and muscle wasting.[4]

DM induces various functional, metabolic and structural changes in the skeletal muscle.[15] In this study, DM is induced by a single intraperitoneal injection of 60 mg/kg BW of STZ. Streptozotocin, a glucosamine-nitrosourea compound, is a genotoxic methylating agent that can selectively destroy insulin-producing pancreatic β-cells through the formation of ROS and DNA alkylation.[16] Oxidative stress is known to occur

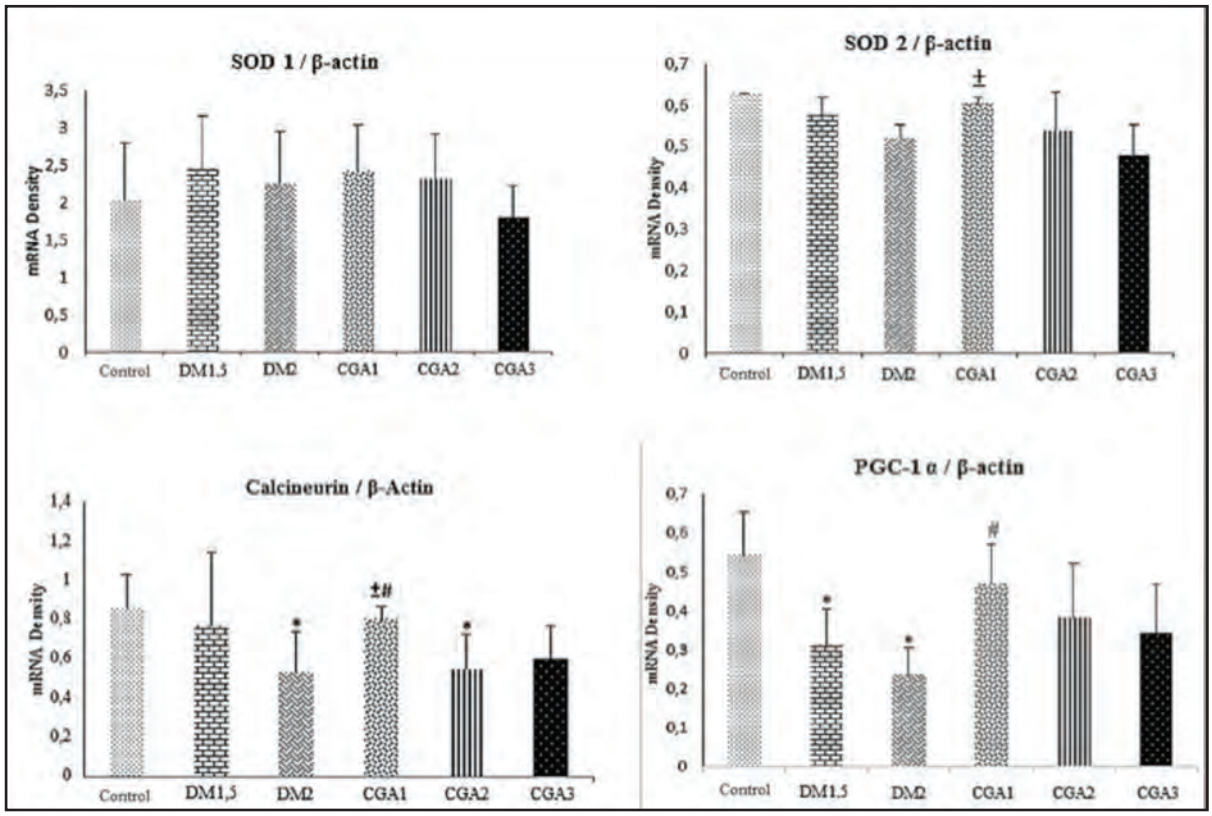

Fig. 3: The mRNA expressions of SOD-1, SOD-2, CN, and PGC-1α in the control, DM, and CGA groups. *: significantly different from control group; #: significantly different from DM2 group; ±: significantly different from CGA3 group

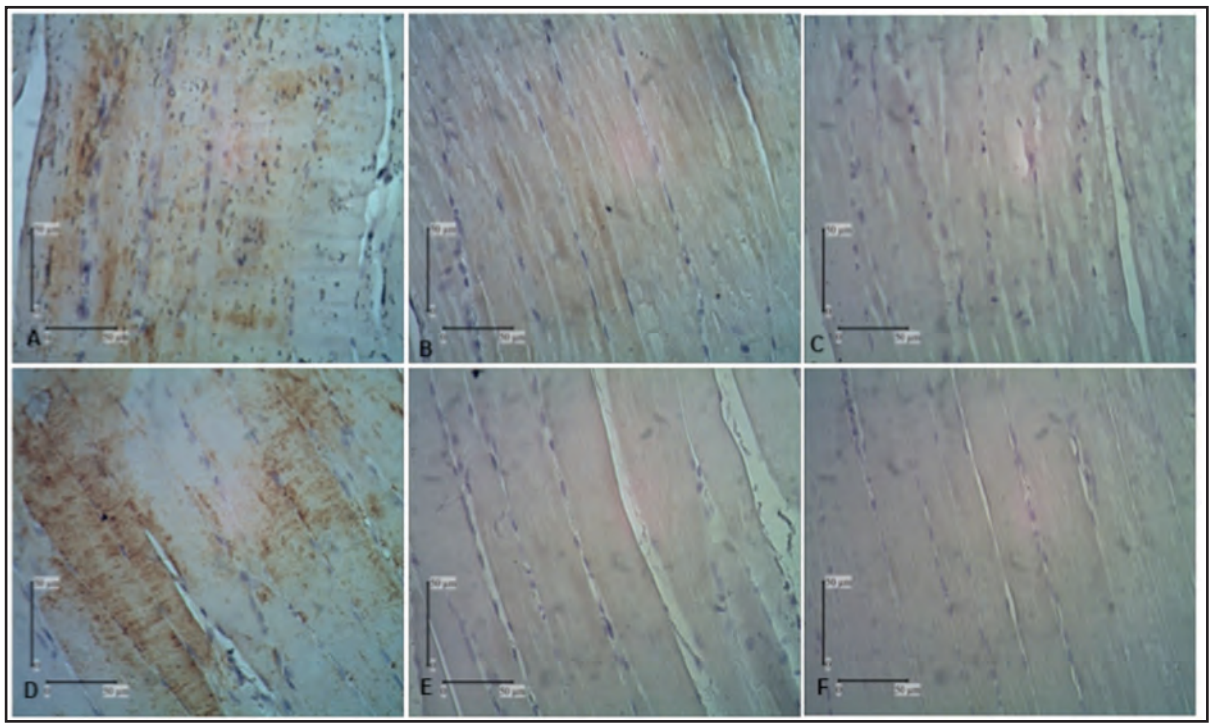

Fig. 4: Calcineurin expression in soleus muscle tissue in the control (A), DM1.5 (B), DM2 (C), CGA1 (D), CGA2 (E), CGA3 (F) groups. Longitudinal sections. Calcineurin-immunohistochemistry showed that immunopositive expression in the CGA1 group was more evident compared to the other groups. Scale bars = 50 μm

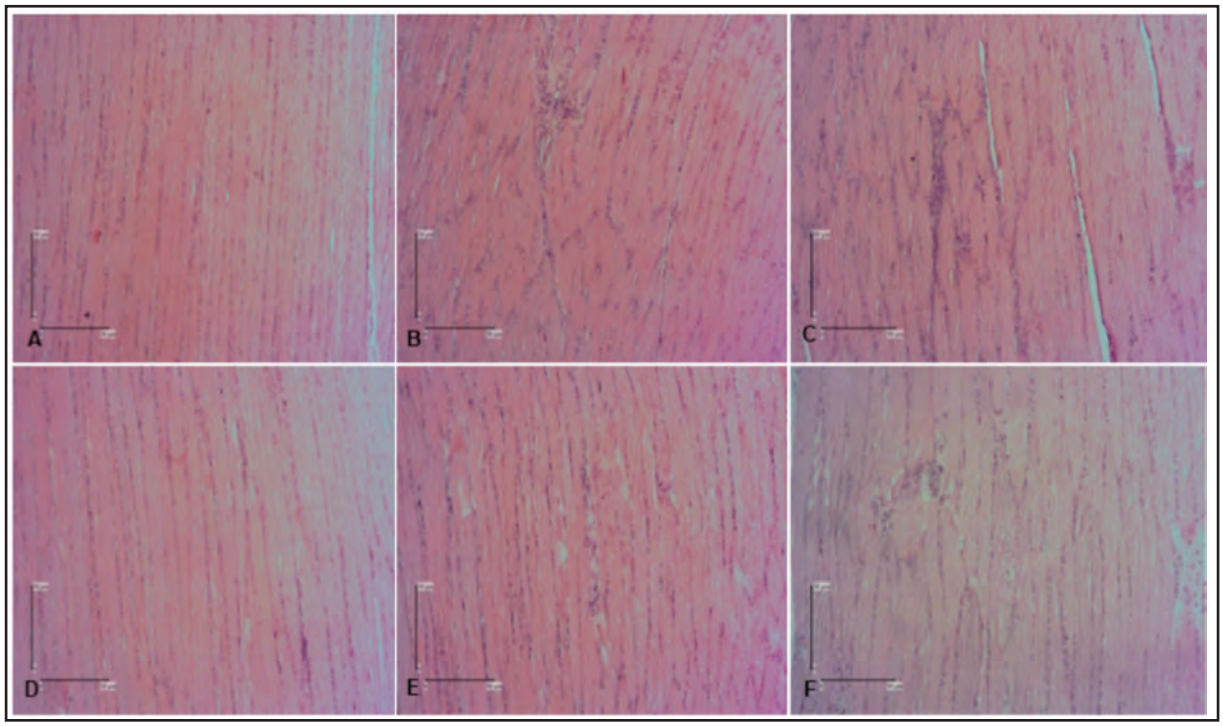

Fig. 5: HE-stained longitudinal sections through soleus muscle tissue in the control (A), DM1.5 (B), DM2 (C), CGA1 (D), CGA2 (E), CGA3 (F) groups. Muscle tissue morphology in the CGA1 group was similar to that in the control group. Scale bars = 50 μm

in both type 1 and type 2 DM and has been directly linked to elevated glucose concentrations.[2] According to the results of blood glucose analysis, it is observed that blood glucose levels in the DM2 group are higher compared to both control and CGA groups. This result is consistent with the previous study suggesting that CGA plays a role in reducing blood glucose levels, thus making it applicable for the prevention and treatment of DM.[17] Chlorogenic acid inhibits glucose uptake from the intestine by suppressing α-glucosidase activity and reducing glucose transport synergistically, ultimately resulting in a decrease in blood glucose levels.1[8]

The activity of the antioxidant defence system in response to increased levels of free radicals due to hyperglycaemia is crucial in DM. Changes in oxidative damage, whether a decrease or an increase, have a significant impact on the process of protein metabolism.[5] Antioxidant enzymes are essential for maintaining a healthy redox state, and one such enzyme that plays a critical role is SOD.[4] In our study, the mRNA expression of SOD-1 is found to be higher in the DM1 group compared to the control group. Presumably, at the onset of DM, SOD-1 increases as an adaptation response to the elevated levels of ROS. However, the mRNA expression of SOD-1 in the DM2 group tends to decrease compared to the control group. This result aligns with previous study that indicates one of the side effects of hyperglycaemia in DM is an increase in ROS production, which in turn heightens susceptibility to oxidative stress.[19]

Antioxidant enzymes such as SOD-1 and SOD-2 are essential for maintaining a healthy redox state.[20] While in the CGA1 group, there is an increase in the mRNA expression of SOD-1 compared to the DM2 group. This result is consistent with previous study showing that CGA offers health benefits by donating hydrogen atoms to reduce free radicals, inhibiting oxidation reactions, and increasing antioxidant enzymes.[21] Additionally, our study shows that mRNA expressions of SOD-2 in the DM groups are also lower compared to the control group, although not statistically significant. These results are in line with previous studies indicating that the increased ROS levels resulting from hyperglycaemia in DM leads to a decrease in antioxidant enzymes, subsequently affecting various signalling pathways.[19] In the CGA groups, there is an increase in the mRNA expression of SOD-2 compared to the DM groups. The CGA1 group exhibits better result with a significant higher level of SOD-2 mRNA expression compared to the CGA3 group. The increase in the mRNA expression of SOD-2 in the CGA groups aligns with previous studies that emphasise the antioxidant benefits of CGA, highlighting its capacity to elevate mRNA expression of SOD-2 in order to maintain a healthy redox balance and reduce ROS.[20]

DM cannot be solely characterised as a disorder of glucose dysregulation, instead, it should be recognised as a chronic inflammation that affects nearly every biological process, including protein metabolism.[13] Changes in protein metabolism and their impact on muscle mass and function are among the least comprehended aspects in the management of DM.[6] Calcineurin is a protein phosphatase 3 and a calcium-dependent serine-threonine phosphatase involved in several adaptive responses that promote muscle fibre growth and regeneration.[22] Our study shows that mRNA expression of CN in the DM2 group is significantly lower compared to the control group. This result is in accordance with previous study indicating that CN levels decrease in DM

condition, marking the initiation of disruptions in various other signalling pathways because CN plays a crucial role in the protein turnover process.[8] Decreased CN activity, resulting from suppression under atrophy-inducing conditions, leads to a reduction in various mediators that are pivotal components of protein degradation in muscle. This, in turn, may contribute to a decrease in muscle functional capacity and muscle mass, which are common companions to DM.[9] In the CGA groups, higher mRNA expression of CN is observed compared to the DM groups. Moreover, mRNA expression of CN in the CGA1 group is significantly higher compared to the DM2 and CGA2 groups. This result aligns with previous study indicating that CGA has the potential to activate and increase CN activity.[20]

In our study, immunohistochemical staining is also performed on muscle tissue sections to visualise CN expression in skeletal muscle tissue. Immunohistochemical examination shows that CN expressions in the DM groups are lower than in the control group. Specifically, CN expression in the DM1 group is lower than in the control group, while in the DM2 group it is lower than in the DM1 group, making it challenging to detect CN gene expression in the DM2 group. These results are in accordance with previous studies demonstrating that CN levels decrease in the context of DM.[8] In the CGA groups, the CGA1 group shows higher CN expression in comparison to both control group and DM groups. Furthermore, the CN expression in the CGA1 group is also higher compared to the CGA2 and CGA3 groups. This pattern of CN immunopositive expression aligns with the results related to the mRNA expression of CN. These results are supported by previous study, which confirms that CGA has the capability to activate and increase CN activity.[23]

This study not only analyses the mRNA expression of CN but also assesses the mRNA expression of PGC-1α in skeletal muscle, which is considered to be closely associated with CN. The result shows that mRNA expression of PGC-1α in the DM groups are significantly lower than in the control group. These findings align with previous study that elucidates the regulation of functional capacity and muscle mass by contractile proteins and calcium signalling activity. One of the pathways known to regulate PGC-1α involves the calcium-dependent phosphatase calcineurin.[24] In the case of DM, the decrease in CN signalling that occurs as a result of reduced PGC-1α regulation, contributing to muscle wasting.[25] In the CGA groups, the CGA1 group exhibits significantly higher mRNA expression of PGC-1α compared to the DM2 group. This result indicates that CGA has the potential to enhance the expression of PGC-1α in skeletal muscle. Our study is consistent with the previous study indicating that CGA can activate and increase CN activity.[25] Increased CN activity is known to promote higher PGC-1α expression in cases of DM.[25]

PGC-1α is a transcriptional coactivator that plays a vital role in regulating skeletal muscle metabolism, particularly energy homeostasis. It achieves this role by controlling glucose transport and is necessary for mitochondrial biogenesis, along with maintaining the oxidative phenotype of muscle fibres.[25] Among the most significant pathways for regulating muscle metabolism is mitochondrial biogenesis. In DM, the process of mitochondrial biogenesis becomes disorganised, leading to a decreased ability of cells to respond to fluctuations in nutrients and energy. This, combined with reduced mitochondrial content and alterations in mitochondrial morphology, is directly linked to the pathogenesis of the disease.[12]

Mitochondria serve various important functions, with their most prominent roles as the primary regulator of cellular metabolic activity by converting energy from macronutrient oxidation into ATP.[24] Mitochondrial activity and function in skeletal muscle are tightly regulated and the process of mitochondrial biogenesis is crucial for preserving mitochondrial integrity.[26] Elevated level of PGC-1α in muscle is linked to enhanced mitochondrial function, increased insulin sensitivity, and the capacity of PGC-1α to stimulate mitochondrial proliferation. This underlines the significance of PGC-1α in the process of mitochondrial biogenesis.[27] Most mitochondrial proteins are synthesised in the nucleus and subsequently targeted to the mitochondria. PGC-1α acts as a direct mediator of transcriptional coactivators involved in cellular energy metabolism and plays an important role in the physiological regulation of protein expression. The protein generated in this process will later be utilized for muscle regeneration, ultimately contributing to increased muscle mass.[20]

CONCLUSSION

The administration of chlorogenic acid (CGA) at a dose of 12.5 mg/kg body weight shows reduced signs of muscle wasting in diabetes mellitus (DM) rats by lowering blood glucose levels, increasing mRNA expressions of superoxide dismutase-1 (SOD-1) and -2 (SOD-2) , calcineurin (CN), and PGC-1α, and improving skeletal muscle structure which similar to that of the control group.

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Vitamin D treatment ameliorates memory function through downregulation of BAX and upregulation of SOD2 mRNA expression in transient global brain ischaemic injury in rats

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ABSTRACT

Introduction: Ischaemic stroke induces oxidative stress with SOD2 downregulation, and BAX upregulation producing apoptosis. Vitamin D is a fat-soluble hormone that has a neuroprotective effect. The aim of this study is to elucidate the role of vitamin D in memory function, oxidative stress and apoptosis in transient global brain schaemic injury (TGBII) model.

Materials and Methods: TGBII was performed in male Wistar rats (3 to 5 months, 150 to 300 g) which underwent bilateral common carotid artery occlusion (BCCAO) for 20 minutes, then reperfused for 10 days (BCCAO group, n = 6). Two groups of BCCAO were treated with intraperitoneal injection of calcitriol 0.125 µg/kgBW (VD1 group) and 0.5 µg/kgBW (VD2 group). The spatial memory function was tested using a probe test with Morris water maze (MWM). mRNA expression of BAX and SOD2 were assessed by the RT-PCR method. Meanwhile, immunohistochemical staining was used for identification of SOD2 protein. Statistical analysis is tested using one-way ANOVA followed by post-hoc LSD.

Results: MWM showed a shorter duration in target quadrant of BCCAO group than the SO group, which is associated with BAX upregulation and SOD2 downregulation. The VD-treated groups had longer duration probe test compared to BCCAO. Furthermore, VD-treated groups had a longer duration in probe test with lower mRNA expression of BAX and higher expression of SOD2. However, there was no significant difference in VD1 and VD2. Immunostaining showed a reduced SOD2 signal in pyramidal cell of CA1 area in BCCAO group and ameliorated in VD1 and VD2 groups.

Conclusion: Vitamin D ameliorates memory function and attenuates oxidative stress and apoptosis in the TGBII model.

KEYWORDS:
Vitamin D, memory function, BAX, SOD2, global cerebral Ischaemic

INTRODUCTION

Stroke is a neurological disease characterised by blockage of blood vessels due to clots that form in the brain and disrupt blood flow, block of arteries and cause the rupture of blood vessels, resulting in the sudden death of brain cells due to lack of oxygen.[1] World Health Organisation (WHO) reported that global heart attack and stroke cerebral Ischaemic is the highest cause of death in the world, more than 6 million stroke cases mortality every year.[2]

Global ischaemic is a condition in which blood flow to all areas of the brain is transiently inhibited, resulting in nerve cell death, known as delayed neuronal cell death. Transient global cerebral ischemia model in rat is induced by bilateral common carotid artery occlusion (BCCAO) that blocked blood flow in carotid arteries using non-traumatic vascular clamps for 20 minutes and then releasing the clamps for reperfusion.[5]

Ischaemic reperfusion injury is a common ischemic stroke, in which blood flow is restored (reperfusion) after an ischemic period. Reperfusion can occur spontaneously, through thrombolytic therapy or mechanically. The main mechanisms of reperfusion injury involved oxidative stress, leukocyte infiltration, mitochondrial mechanisms, platelet activation and aggregation, complement activation and disruption of the blood brain barrier (BBB),[6] that will disrupt spatial learning and memory processes.[8]

The cornu ammonis region 1 (CA1) is a pyramidal cell in the hippocampus that is more susceptible to damage and delayed neurological death than the CA3 region when oxygen is deprived. Disruption of CA1 neurons contributes to memory deficits in patients with hippocampal injury.[9] Brain ischemia involves multiple mechanisms, such as excitotoxicity, mitochondrial dysfunction, oxidative stress, inflammation, autophagy and damage to the BBB.[10]

Oxidative stress and neuronal apoptosis are important factors in the pathological process of ischemic reperfusion injury in cerebral ischaemic stroke followed by reperfusion. Oxidative stress refers to a comparative surplus of reactive

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oxygen species (ROS) that is caused by an imbalance between oxidants and antioxidants.[11] Brain tissue is sensitive to oxidative stress because it contains low levels of endogenous antioxidant enzymes, including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT), which act as cellular defences against ROS. Oxidative stress is involved in the initiation of apoptosis, furthermore a balance between the anti-apoptotic B cell lymphoma protein (Bcl-2) and the pro-apoptotic regulatory protein BAX regulates apoptosis. BAX mediates caspase-9 activation which upregulates in human brain tissue after ischemia. In ischemic stroke animal, caspase-9 leads to the activation of caspase-3 as the main mediator of apoptosis.[11]

Vitamin D is commonly associated with calcium and bone metabolism, but recently it was found that it is an important component in the development of nerve dysfunction.[12] Vitamin D is an antioxidant in an active form called (calcitriol or 1,25-dihydroxy vitamin D3) and has a neuroprotective effect.[13] Vitamin D can maintain a balance between free radicals and antioxidants by increasing intracellular antioxidant concentrations and eliminating excess free radicals thereby reducing oxidative stress.[14] Vitamin D can suppress BAX activity in the hippocampus, as well as caspase-3 activity through the intrinsic pathway of apoptosis where calcitriol provides a neuroprotective function mouse model of global cerebral ischaemic (GCI).[15] Vitamin D has a neuroprotective effect on hippocampal apoptosis induced by pentylenetetrazole and kainic acid in rats.[16]

Administration of vitamin D increases the expression of ET-1, eNOS and triggers the repair of vascular remodelling in the renal fibrosis model mouse.[17] Stroke patients with low vitamin D levels have a wider infarct volume and worse functional effects.[18] In previous study, it was found that from 30 stroke patients, 65% had decreased vitamin D and 35% had normal vitamin D.[20] Existing studies have not provided an overview of the effect of vitamin D on ischemia conditions in terms of changes to memory, oxidative stress and apoptosis. Therefore, this study was conducted to determine the effect of vitamin D in ischaemic conditions on spatial memory, the apoptotic marker BAX and the antioxidant enzyme SOD2.

MATERIALS AND METHODS

Animal Handling and Transient Global Brain ischaemic Injury Model

The study used 24 male Wistar rats (Rattus norvegicus) aged 2 to 3 months with 150 to 300 g body weight obtained from the University of Muhammadiyah Yogyakarta. This research was approved by the Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Gadjah Mada University on November 24, 2022 with number KE/FK/1486/EC/2022. Rats were randomly divided into four groups (n = 6): SO (Sham operation), BCCAO (transient global cerebral ischemia without vitamin D), VD1 (transient global cerebral ischemia + 0.125 µg/kg/day ip vitamin D injection) and VD2 (transient global cerebral ischemia + 0.5 µg/kg/day ip vitamin D injection).

BCCAO was performed to induce transient brain ischemia. The protocol was based on previous studies.[5,18,19-23] Briefly, the rats were anesthetised using 100 mg/kgBW of ketamine anaesthesia (PT Guardian Pharmatama, Jakarta, Indonesia). During anaesthesia, the anterior midline of the necks of the rats was opened and the common carotid arteries were exposed and clamped using non-traumatic vascular clamps (Dieffenbach, World Precision Instruments, USA). The clamps were left obstructing the blood flow of both common carotid arteries for 20 minutes. Sham operations were carried out on the rats of the SO group. After the surgery, the rats were kept in the recovery phase for 2 days before being tested for spatial memory. The rats were sacrificed on day 10.

Vitamin D Administration

This study used calcitriol (1,25-dihydroxy vitamin D) as active vitamin D in a crystalline solid preparation dissolved in 0.2% ethanol to obtain a concentration of 1 mg/ml. Vitamin D was given at different doses, namely 0.125 µg/kg BW in group 3 and 0.5 µg/kg BW in group 4 by intraperitoneal injection once per day until the rats were determined.

Morris Water Maze Test Probes

Rats were tested for memory with a probe test in a Morris water maze (MWM) for 5 days. From the first day until the fourth day, the rats were allowed to practice finding a foothold (platform) in a fixed position in the MWM pool four times a day then dried and returned to the cage. On the fifth day, the rats were subjected to a probe test, and allowed to swim to find a foothold for 2 minutes, without foothold. As long as the rats are looking for a foothold, the camera is provided to records data on the rat's long track for searching the target quadrant/quadrant 4 (Q4).

Animal Termination and Hippocampus Harvesting

The rats were terminated at 10th day after BCCAO procedure. The rats were anesthetised with ketamine HCl 80 to 100 mg/kgBW by intramuscular injection. After being deeply anesthetised, rats were positioned supine on the operating table, then the abdominal wall was incised from the median line to the right and left lateral sides to open the abdominal and thoracic cavities. The organ perfusion was carried out by flowing 0.9% NaCl solution into the left ventricle. The perfusion process has waited until abdominal visceral organs turned pale, and then the rats were decapitated. The cerebrum is separated from the cranium by cutting the sagittal suture so that the two hemispheres are separated. The dextra hippocampus was separated from the dextra cerebral hemispheres then it was put into RNA later and stored in the refrigerator at -20°C. The left cerebral hemispheres were soaked in 10% formalin in PBS for 24 hours and continued with making paraffin blocks.

Immunohistochemistry SOD2

Four paraffin blocks containing hippocampal tissues were deparaffinised, heated in citrate buffer solution for 20 minutes, and incubated in 3% H2O2 in methanol for 15 minutes. Blocking non-specific antigen are performed using background sniper from the Starr Trek HRP Universal Detection Kit (Biocare Medical®, USA, Cat. #STUHRP700H). Afterwards, the sections were incubated in primary anti-

SOD2 antibody (Bioss, Cat. #bs-1080R, 1:100), overnight at 4°C. On the following day, the sections were incubated in appropriate secondary antibodies for an hour and incubated with avidin-HRP from the Starr Trek HRP Universal Detection Kit (Biocare Medical®, USA, Cat. #STUHRP700H) for 30 minutes. Hippocampal pyramidal cell images are viewed under a digital camera (Optilab, Miconos, Indonesia) connected to a light microscope (Olympus, USA) and a computer with 400x magnification. SOD2 positive cells are brown in the cytoplasm.

RNA Extraction and cDNA Synthesis

The right hippocampus previously kept in RNA later® Stabilization Solution (Thermo Fisher Scientific, USA, Cat. #AM7021) was cut in half. Hippocampal tissue RNA was extracted using the Smobio KIT. The cDNA synthesis process was carried out according to the protocol of ExcelRT™ Reverse Transcription Kit II (Smobio®, Cat. No. RP1400) using extracted RNA. The cDNA results were stored in a refrigerator at -20oC. 3 µl cDNA was mixed with a PCR mixture consisting of 0.8 µl forward primer, 0.8 µl reverse primer, 12.5 µl GoTaq™ Green Master Mix, and 7.9 µl nuclease-free water. The following primers were used (Table I).

RT-PCR machine configuration following initial denaturation 95°C for 2 minutes, denaturation 95°C for 10 seconds, annealing 57.60°C for 1 minute, elongation 72°C for 1-minute, last extension 72°C for 10 minutes, and hold 4°C for 40 cycles. The PCR products were analysed by electrophoresis on 2% agarose gel (Agarose S; Nippon Gene, Tokyo, Japan). The electrophoretic results were photographed by transillumination with ultraviolet light using the Geldoc Syngene Gbox Chemi XRQ Series, then densitometry analysis was measured with ImageJ®.

Analysis of Results and Statistical Tests

Statistical analysis in this study used IBM SPSS Statistics 22 software. The data normality test used the Shapiro-Wilk test for a sample size of less than 50. One-way ANOVA followed by LSD post-hoc multiple comparison analysis was used to examine the mean differences between groups - research subject groups. The mean difference is said to be statistically significant if the p-value < 0.05.

RESULTS

Vitamin D Improved Spatial Memory Function Based on MWM Assessment of Length of Time and Trajectory in the Probe Test

The spatial memory of rats after transient brain ischaemic was evaluated using the MWM procedure consisting of escape acquisition, memory retention and visible platform tests. Probe test analysis revealed that the BCCAO group had a shorter average time in the target quadrant than the SO group. The VD1 and VD2 groups had an average longer time in the target quadrant than the BCCAO group (Figure 1A). One-way ANOVA test revealed that the BCCAO group was significantly different from the SO group ($p = 0.028$), which indicated the memory function in the BCCAO group was worse than the control group. The VD1 group ($p = 0.004$) and the VD2 group ($p = 0.017$) had higher time results and significantly different compared to the time in the BCCAO group. Meanwhile, there was no statistical difference between the two groups given vitamin D.

In the trajectory image, the SO group focused more on the target quadrant, in contrast, in the BCCAO group, the rats circle more around other quadrants than the target quadrant and do not appear to have a search focus on the target quadrant but almost all quadrants are surrounded by rats. In the VD1 and VD2 groups, the rats circled the target quadrant more, and the rats seemed to have a more focused search on the target quadrant (Figure 1B).

Vitamin D Downregulated Expression of BAX and Upregulated Expression of mRNA SOD2 and SOD2 Immunohistochemical Staining

Examination of both mRNA expression of BAX and SOD2 and SOD2 immunohistochemical was assess the effect of vitamin D on apoptotic regulatory markers and antioxidant enzymes. RT-PCR analysis revealed that there is a significant difference between all the groups. The LSD post hoc test showed the expression of BAX mRNA in the BCCAO group ($p = 0.000$) higher than the SO group. Meanwhile, the expression of BAX mRNA in the VD1 ($p = 0.029$) and VD2 group ($p = 0.007$) was lower than in the BCCAO group (Figure 2B). In addition, the LSD post hoc test showed that SOD2 mRNA expression in the BCCAO group ($p = 0.008$) was lower than in the SO group, while SOD2 mRNA expression in the VD1 group ($p = 0.011$) and VD2 ($p = 0.016$) was higher than the BCCAO group (Figure 2C).

DISCUSSION

The main finding of this study is transient brain ischemia using the BCCAO model significantly reduces the memory function that affects the spatial learning ability of rats. This corresponds to a decrease in BAX mRNA expression and an increase in SOD2 mRNA expression.

The BCCAO surgical procedure successfully induces temporary brain ischemia, which causes a decrease in spatial memory function. This finding is similar to several studies that showed memory deficits on the MWM test and damage to the CA1 area of the hippocampus due to BCCAO

Table I: List of primers used in this study

Gen	Primary	No. catalogue
SOD2	F: ATGTTGTGTCGGGCGGCGTGCAGC	IDT - 97228330; 200806011
	R: GCGCCTCCGTGGTACTTCTCTCCGGTG	IDT - 97228329; 200806012
BAX	F: GTGAGCGGCTGCTTGTCT	IDT - 97228333; 180583538
	R: GGTCCCGAAGTAGGAGAGG	IDT - 97228366; 180583539
GAPDH	F: GTTACCAGGGCTGCCTTCTC	IDT - 98517206; 205392190
	R: TCCCGTTGATGACCAGCTTC	IDT - 98517207; 205392191

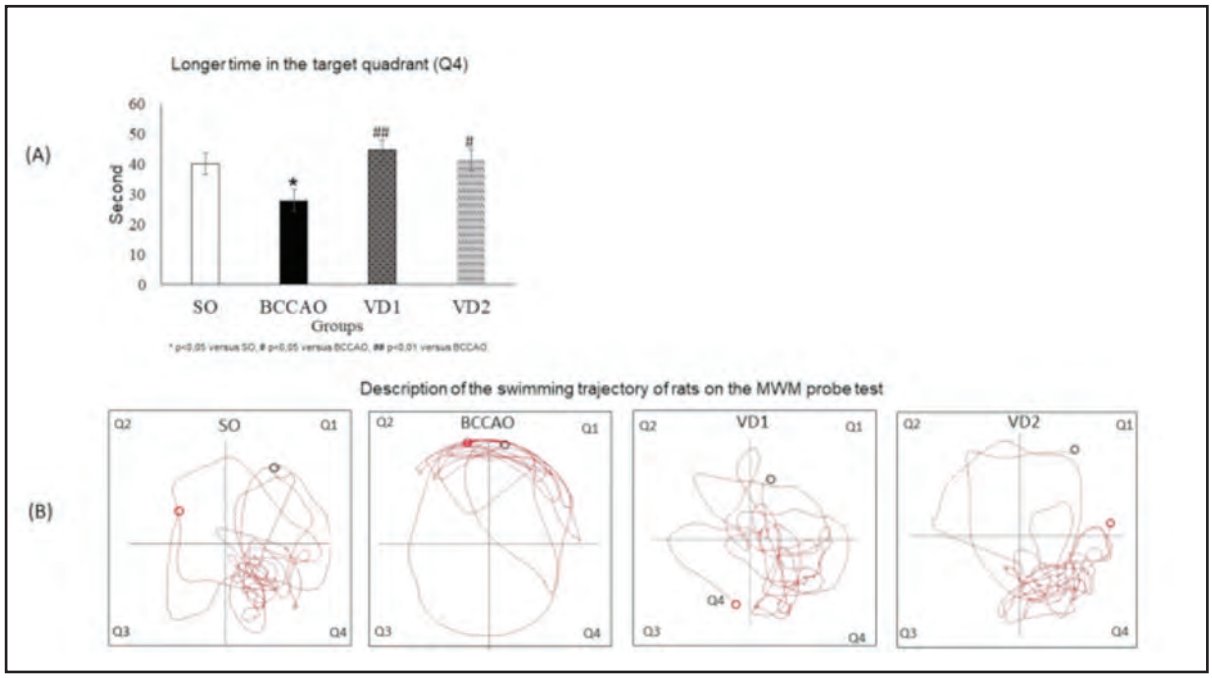

Fig. 1: Graphical representation of the probe test and the probe test path. (A) Graph of the length of time rats spent in the target quadrant (Q4) and (B) Overview of mouse probe test pathways (SO, BCCAO, VD1, VD2)
Notes: (A) * p < 0,05 vs SO, # p < 0.05 vs BCCAO, ## p < 0.01 vs BCCAO. (Analysed by one-way ANOVA, post-hoc LSD test); (B) Target quadrant: quadrant 4 (Q4);O: swimming starting point; O: finishing point of swimming

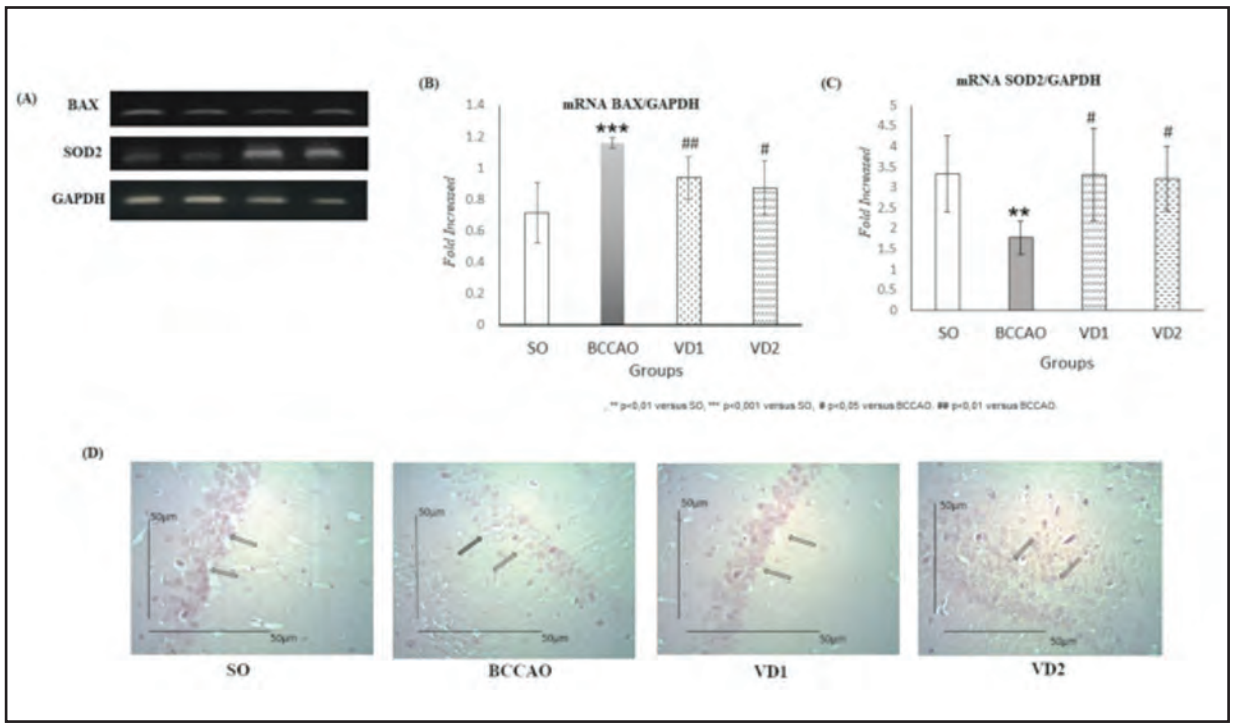

Fig. 2: (A) Representative images of the electrophoresis results of RT-PCR products. (B) Comparison of mean ± SD of BAX/GAPDH mRNA expression. (C) SOD2/GAPDH mRNA expression between the 4 groups of experimental animals. (D) Representative immunohistochemical staining images of the SOD2 enzyme in pyramidal cells of the hippocampal CA1 region. Arrows indicate the expression of the antioxidant enzyme SOD2 as brown spots in the pyramidal cells of the CA1 region of the hippocampus.
Notes: (B, C) ***p < 0.001 vs SO; # p < 0.05 vs BCCAO; ## p < 0.01 vs BCCAO; (Analysed by one-way ANOVA, post-hoc LSD test); (D): Magnification: 400x. ×; Scale bars 50 µm

procedure.[5,23,26-30] It was observed that the learning performance of rat from the ischemic group (BCCAO) tended to be worse than the SO group on the MWM probe test (Figure 1). The decreased spatial memory in rats likely stems from damage to the CA1 region of the hippocampus which is more severely affected by transient brain ischaemic. Therefore, several studies have reported that the CA1 region is more susceptible than the CA2-CA3 region in response to ischaemic.[5,31-33] Transient global cerebral ischemia induced by the BCCAO technique causes a detrimental effect on the brain called reperfusion injury.[6] Reperfusion ischemia injury results in increased production of free radicals which will induce apoptosis of neuron cells, especially in vulnerable areas, namely the hippocampus.[4,5]

The results of the probe test show that the VD1 and VD2 groups had a better memory retention function in the MWM probe test compared to the BCCAO group. Based on the trajectory pattern of the rats in the memory retention test, it was found that the SO rats swam more directionally, heading straight for the target quadrant and swimming more in the target quadrant locations. BCCAO group swam thematically, and the rats have difficulty finding the target quadrant's location. Rats in the VD1 and VD2 groups swam more directionally, finding the target quadrant and swam more in the target quadrant locations (Figure 1A). Based on the pattern of the swimming trajectories of the rats in this study, the SO group and the VD-treated groups seemed to be swimming using the hippocampus-dependent allocentric swimming strategy. In contrast, the BCCAO group appeared to use the hippocampus-independent egocentric swimming strategy (Figure 1B).[34] This suggests that global cerebral ischemia induction performed by the BCCAO technique disrupts spatial memory function according to previous studies.[5,8,35,36]

Different doses are given to determine the effect of the dose. At both doses, there is no significant difference in improving spatial memory function after transient global cerebral ischemia. This aligns with other studies which compared 0.125 µg/kgBW, 0.25 µg/kgBW and 0.5 µg/kgBW doses of vitamin D on fibroblast expansion, inflammation and apoptosis of kidney epithelial cells in UUO models and found that there was no significant difference between the three dose groups.[17] The group that was given vitamin D had a spatial memory function that comparable to the control group which did not undergo ischemia (SO). This demonstrated that vitamin D could restore spatial memory function in global cerebral ischemia until comparable to normal conditions.[37]

A low diet of vitamin D impairs spatial memory function in adult rat. Improvement of spatial memory function in the transient global cerebral ischemia VD-treated showed that vitamin D can induce protection against oxidative stress by upregulating antioxidant proteins.[25,38]

In this study, BAX mRNA expression in the BCCAO group was higher than in the SO group. While BAX mRNA expression in VD1 and VD2 was significantly lower than BCCAO group (Figure 2B). During cerebral ischemia, an imbalance of ionic gradients occurs, depolarising neurons and causing neurotransmitter release, which leads to increased accumulation of glutamate in the extracellular space. Glutamate activates ionotropic glutamate receptors (N-methyl-D-aspartate receptors/NMDAR), which act as excitotoxic channels and allow entry of glutamate ions. Bond of glutamate to NMDAR affects ischaemic-induced Ca^{2+}, which impacts the increased intracellular Ca^{2+} and accumulation of ROS, activating calpains and mediating the cleavage of Bid to tBid, which integrates the different death pathways. At the mitochondrial membrane, tBid interacts with BAX, and BAX forms pores in the mitochondrial outer membrane, releasing Cytc, which executes caspase-dependent cell death. Upon release into the cytosol, the Cytc and procaspase-9 complex form the apoptosome that activates execution caspase, such as caspase-3.[39] Administration of vitamin D protects nerve cells by preventing cytotoxicity and apoptosis, and downregulating L-type voltage-sensitive calcium channels A1C (LVSCC A1C) and upregulating VDR. Vitamin D administration protects against glucocorticoid-induced apoptosis in hippocampal cells, representing vitamin D-mediated neuroprotection.[40]

Calcitriol or 1,25-dihydroxyvitamin D3 is a metabolite of the active form of vitamin D in the kidney.[17] Vitamin D has a mechanism as a neuroprotection that can reduce eruptions and affect complications after a stroke. Another study reported that serum 25(OH)D concentration was inversely related to ischemic infarction volume. In previous studies, vitamin D protects nerves from ischaemic, including postischemic inflammatory response.[3] Vitamin D can suppress BAX activity in the hippocampus, and caspase-3 activity through the intrinsic pathway of apoptosis, where calcitriol provides a neuroprotective function in the mouse model of global cerebral ischemia (GCI).[15] Vitamin D has a neuroprotective effect on hippocampal apoptosis induced by pentylenetetrazole and kainic acid in rat.[16]

The mechanism of vitamin D that can reduce the neurotoxic effects due to oxidative stress can be explained through the following three mechanisms: First, the protective effect of antioxidants against ethanol-induced oxidative stress may stem from the modulation of the expression of survival-enhancing molecules, for example the BCL-2 gene family.[4] Several studies proved that survival-enhancing proteins, such as Bcl-2 and Bcl Xl, play a role in the antioxidant pathway to inhibit apoptosis and oxidation processes, such as lipid peroxidation. Second, vitamin D acts as a membrane antioxidant that protects neurons from damage caused by oxidative stress. Vitamin D accumulates in cell membranes and reduces lipid peroxidation. Several studies suggest that the antioxidant function of vitamin D may be more potent than vitamin E, melatonin and oestrogen. Third, vitamin D has a protective effect by regulating Ca^{2+} homeostasis in brain cells. Vitamin D is known to influence Ca2+ uptake in some inducible cells and to modulate voltage sensitive Ca^{2+}. This demonstrated vitamin D may have a potent neuroprotective effect against glutamate-mediated cytotoxicity.[4,25]

In this study, the expression of SOD2 mRNA in the BCCAO group was lower than the SO group. In contrast, the expression of SOD2 mRNA in the VD1 and VD2 groups was

higher than the BCCAO group (Figure 2C). This study revealed that the localisation of SOD2 expression in the BCCAO group was less than the SO group. SOD2 expression in the VD1 and VD2 groups did not appear to be more than in the BCCAO group. Long-term administration of vitamin D decreases the expression of caveolin-1 (Cav1/L-VGCC subunit) which reduces calcium influx in response to ROS, inflammation and stress.[7] ROS causes translocation of NF-kB to the nucleus and produces proinflammatory cytokines (TNFα, IL-6, IL1β). 1,25(OH)2D3 plays a role in inhibiting NF-kB translocation from the cytoplasm to the nucleus impacting NF-kB expression, reducing iNOS expression and reducing NO production.[7,25]

Previous research stated that vitamin D increases the expression of glutathione peroxidase, which converts ROS H_2O_2 molecules into water. Vitamin D affects the formation of glutathione by the activating the enzyme glucose-6 phosphate dehydrogenase, downregulating NOX to produce ROS, converting O_2 to H_2O_2 and increasing SOD. Vitamin D administration collectively reduces the intracellular burden of ROS.[24] Increased SOD expression protects against brain damage in cerebral ischemia by reducing oxidative injury and modifying redox signals. SOD also reduces mitochondrial dysfunction and subsequent apoptosis after cerebral ischemia. In contrast, NOX, a pro-oxidant enzyme, exacerbates cerebral oxidative stress and contributes to ischemic brain damage.[20]

Vitamin D can maintain the balance between free radicals and antioxidants by increasing intracellular antioxidant concentrations and eliminating excess free radicals thereby reducing oxidative stress.[15] Stroke patients with low vitamin D levels have a more expansive infarct volume and worse functional effects. Another study reported that the serum concentration of 25(OH)D was inversely related to ischemic infarction volume.[3] In previous studies, it was stated that vitamin D protects nerves after ischaemic, inflammation.[3]

CONCLUSSION

Vitamin D has neuroprotective effect on the hippocampus and also ameliorates memory function through downregulation of BAX mRNA expression and upregulation of SOD2 mRNA expression in transient rat models of global cerebral ischemia. Vitamin D could improve antioxidant defence and reduce neuronal apoptosis, thereby preserving memory function.

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ORIGINAL ARTICLE

Comparing sedative and non-sedative reduction techniques in paediatric intussusception: Insights from a 6-year study

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ABSTRACT

Introduction: Intussusception is a prevalent paediatric emergency condition. The standard of care involves the reduction using air or fluid enema is considered a safe procedure. Sedation-induced muscle relaxation thus optimising the treatment. We present a comprehensive 6-year study involving non sedative reduction (NSR) versus sedative reduction (SR) utilising ketamine and midazolam.

Materials and Methods: A retrospective cohort study was conducted between January 2017 and July 2023 in Yogyakarta, Indonesia. A total of 85 children diagnosed with intussusception underwent hydrostatic reduction, which employed water-soluble contrast administered into the rectum. Cases that were unsuccessful in reduction underwent immediate surgical intervention.

Results: Among the 85 children with intussusception underwent reduction, 22 children underwent the SR procedure and 63 underwent NSR procedure. We found a successful outcome in 17 cases (77%) of SR procedure with one recurrent and the other five (23%) got surgical reduction such as anastomosis resection (3 cases) due to Meckel-Diverticula. On the other hand, we found 24 successful cases (38.0%) in NSR procedure with one recurrent after case. 39 others who failed with NSR continued to surgical reduction. Manual reduction was done for 31 patients with one case mortality due to pulmonary bleeding. Anastomosis resection (4 cases) and, stoma (4 cases) were decided for others surgical reduction. The relative risk (RR) on this study was 2.02 (p value < 0.05, CI 95%).

Conclusion: Implementation of the SR procedure may reduce surgery rates in paediatric intussusception, thereby enhancing patient management. Furthermore, the success rate of hydrostatic reduction higher in under sedation procedure. We contribute to evolve insight of non-operative approaches of paediatric intussusception management, particularly in the Yogyakarta.

KEYWORDS:
Paediatric, intussusception, hydrostatic reduction, sedative

INTRODUCTION

Intussusception is a prevalent paediatric emergency condition and the main cause of bowel obstruction in children aged less than 5 years, which is an invasion of the proximal bowel into the distal bowel. The structures frequently involved in this condition are the small intestine and colon. Ileocolic intussusception is the most typical type of intussusception, with ileoileocolic, enteroenteric and colocolic intussusceptions occurring less frequently.[1] Its early identification and care are crucial because late diagnosis can result in ischemia, potentially leading to necrosis, perforation and peritonitis to death due to septic shock.[2-4] The best diagnostic method is transabdominal ultrasonography because of its high sensitivity (98%), safety and accessibility. The standard of care for intussusception involves the reduction using air or fluid enema is considered a safe procedure.[4] Hydrostatic reduction (HR) through the anorectal route offers a surgical risk-free alternative. While there has been ongoing debate regarding the efficacy of employing sedation, recent research has indicated that sedative reduction can be a successful method for treating intussusception. This may be attributed to its ability to induce smooth muscle relaxation, allowing healthcare providers to secure patient cooperation while minimizing movements and cries.[5] We present a comprehensive 6-year study involving non sedative reduction (NSR) versus sedative reduction (SR) utilising ketamine and midazolam.

MATERIALS AND METHODS

Patients and Methods

This research is a retrospective cohort study to compare the effectivity of non-sedative reduction (NSR) versus sedative reduction (SR) utilising ketamine and midazolam to reduce surgery rates in paediatric intussusception. A total of 85 children with intussusception with hydrostatic reduction were included in the study, conducted between January 2017 and July 2023 at Sardjito Hospital, Yogyakarta, Indonesia. Data included patient age, sex, body weight, vomiting, bloody stools, abdominal distention, symptom duration, location intussusception and recurrence of intussusception were collected and analysed during a retrospective chart review. Patients who had unstable hemodynamic, peritonitis, pneumoperitoneum at the time of their initial presentation

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Table I: Characteristic of children with intussusception in each treatment group

Characteristics	Treatment groups		
	Sedative (mean ± SD)	Non-sedative (mean ± SD)	p-value
Age (mo)	25 ± 14.38	17.14 ± 12.29	0.136
Weight (kg)	11.38 ± 4.45	9.08 ± 2.39	0.012*
Duration of clinical manifestation (h)	80.57 ± 41.42	75.27 ± 42.21	0.765

	Sedative		Non-sedative		p-value
	n	%	n	%	
Gender					0.459
Male	12	54.5	40	63.5	
Female	10	45.5	23	36.5	0.459
Clinical manifestation					
Vomiting	21	95.5	50	79.4	0.08
Red currant jelly stools	12	54.5	39	61.9	0.544
Abdominal distension	8	36.4	27	42.9	0.594
Location					0.07
Right	14	63.6	26	41.3	
Left	8	36.4	38	58.7	

Table II: Comparison of reduction successful rate between groups

		Reduction successful rate						p-value	RR	95% Confident interval
		Success		Failed		Total				
		n	%	n	%	n	%			
Procedure	Sedation	17	77.3	5	22.7	22	100	0.002	2.028	1.376 – 2.990
	Non sedation	24	38.1	39	61.9	63	100			
	Total	41	48.2	44	51.8	85	100			

underwent immediate surgery and were not included in this study.

A supine position was used to perform hydrostatic reduction on the patient, and water-soluble contrast was inserted through an anorectal tube at the level of 1 meter of hydrostatic pressure. The radiologist monitored the passage of fluid through the intussusception under fluoroscopy guiding. Ketamine (1 mg/kg/dose) and midazolam (0.1 mg/kg/dose) were delivered intravenously 5 minutes prior to the attempt at hydrostatic reduction in the patient undergoing the SR procedure. Throughout the procedure, the patient's state of consciousness, breathing rate, heart rate and blood oxygen levels were closely observed due to the potential risk of apnoea or respiratory cessation. If the chosen approach proved ineffective, prompt surgical intervention under general anaesthesia was pursued following the acquisition of written informed consent.

Statistical Analysis

SPSS 26.0 for Windows (IBM, Chicago, IL, United States) was used for the statistical analysis. Patients were divided into two groups who underwent SR procedure (SR group) and NSR procedure (NSR group). Data were expressed as mean ± standard deviation or number and percentage. Chi-square test was used to analyse risk factors contributing to HR failure. Statistical significance was set as p value < 0.05. We analyse the risk estimate between two groups.

RESULTS

The mean age of the patient was 25 ± 14.38 months in the SR group and 17.14 ± 12.29 months in the NSR group, the mean weight was 11.38 ± 4.45 kg and 9.08 ± 2.39 kg in SR groups and NSR groups respectively. The mean duration of clinical manifestations was 80.57 ± 41.42 and 75.27 ± 42.21 hours in the SR group and NSR group respectively. Most of the children in both groups were male, 12 (54.5%) and 40 (63.5%). Vomiting, bloody stools and abdominal distension were observed in 21 (95.5%), 12 patients (54.5%) and eight (36.4%) patients in the SR group respectively. In the NSR group, vomiting, bloody stools and abdominal distension were observed in 50 (79.4%), 39 patients (61.9%) and 27 (42.9%) patients. Based on the location of the abnormalities, in the SR group most (63.6%) of the abnormalities occurred in the right side of intestine, while in the NSR group, most of the abnormalities were located in the left side of intestine, namely in 38 patients (58.7%) (Table I).

In this study, among 85 children with intussusception who underwent hydrostatic reduction, 22 children underwent the SR procedure and 63 underwent the NSR procedure. Among 63 children who underwent the NSR procedure, we found 24 successful cases (38.0%) of the NSR procedure with one recurrent after case. 39 others who failed with NSR continued to surgical reduction. Manual reduction was done for 31 patients with one case of mortality due to pulmonary bleeding. Anastomosis resection and stoma were performed in each of the other four patients. On the other hand, in the

Table III: Baseline characteristics of children with EA in our institution

Characteristics	n (%)
Sex	
• Male	28 (52.8)
• Female	25 (47.2)
Weight at diagnosis (gram)	
• Normal weight (≥ 2500)	22 (41.5)
• Low weight (< 2500)	28 (52.8)
• Very low weight (< 1500)	3 (5.6)
• Extremely low weight (< 1000)	0
Gestational age	
• Preterm	15 (28.3)
• At term	11 (20.8)
• Post-term	27 (50.9)
EA type	
• Isolated EA without TEF (Gross A)	9 (17)
• EA with distal TEF (Gross C)	44 (83)
Thrombocytopenia (< 150,000/mm3)	
• Yes	31 (58.5)
• No	22 (41.5)
Pneumonia	
• Yes	48 (90.6)
• No	5 (9.4)
Sepsis	
• Yes	50 (94.3)
• No	3 (5.7)
Definitive surgery (oesophageal anastomosis)	
• Yes	17 (32.1)
• No	36 (67.9)
Outcome	
• Survived	10 (18.9)
• Died	43 (90.1)
Associated anomaly	
• VACTERL	27 (51)
• VACTERL, undescended testis	1 (1.9)
• VACTERL, Opitz G/BBB syndrome	1 (1.9)
• VACTERL, Down syndrome, clubfoot	1 (1.9)
• VACTERL, Meckel diverticulum	1 (1.9)
• VACTERL, hypospadias, undescended testis, left radial clubhand	1 (1.9)
• VACTERL, dextrocardia	2 (3.8)
• VACTERL, cholestasis	1 (1.9)
• Tracheomalacia	1 (1.9)
• No associated anomaly	14 (26.4)
• Unknown	3 (5.7)

EA: Oesophageal atresia; TEF: Tracheoesophageal atresia

Table IV: Multivariate analysis of survival of children with EA in our institution

Variables	HR (95% CI)	p-value
Sex	0.82 (0.42–1.63)	0.578
EA type	0.69 (0.23–2.02)	0.496
Thrombocytopenia	2.67 (1.22–5.85)	0.014*
Pneumonia	3.67 (0.84–16.04)	0.084
Sepsis	0.80 (0.12–5.41)	0.817
Definitive treatment	0.39 (0.17–0.87)	0.022*

*, $p < 0.05$; CI: Confidence interval; HR, hazard ratio; EA, oesophageal atresia

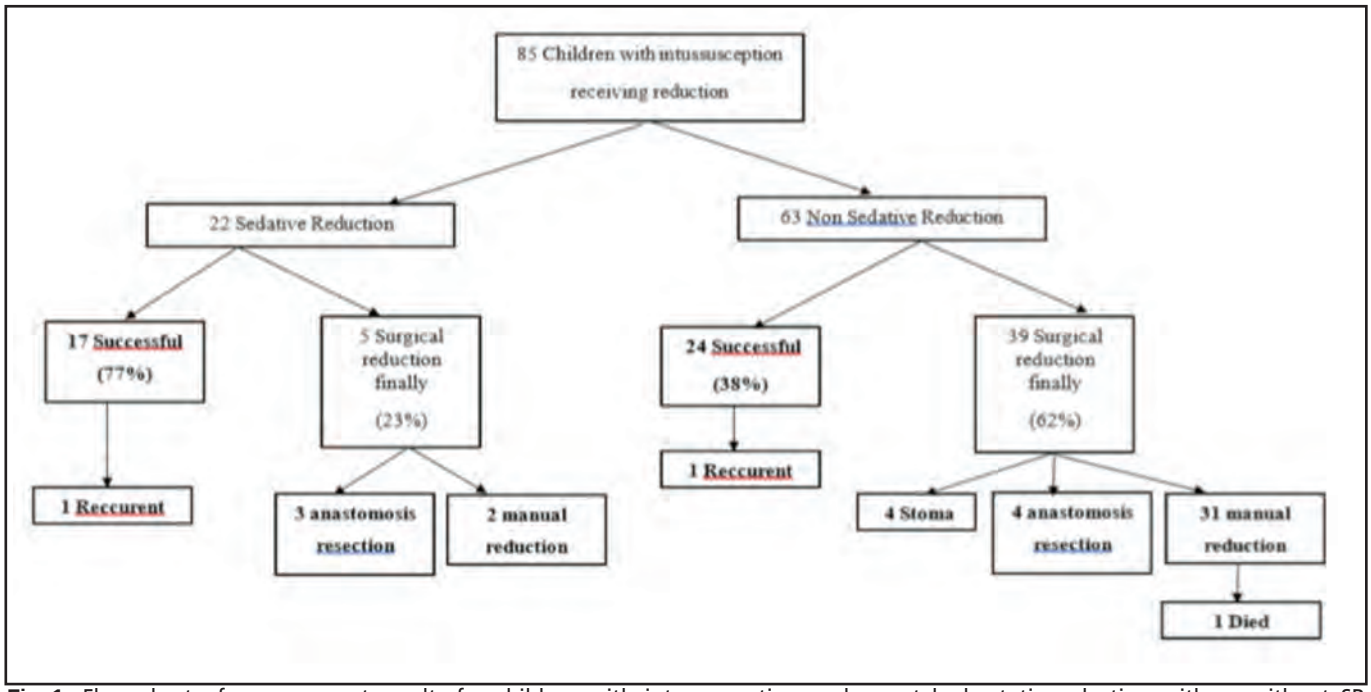

Fig. 1: Flow chart of management results for children with intussusception underwent hydrostatic reduction with or without SR procedure

22 children who underwent the SR procedure, we found a successful outcome in seventeen cases (77%) of the SR procedure with one recurrent. The other five (23%) got surgical reduction such as anastomosis resection(3 cases) due to Meckel-Diverticula (Figure 1).

Based on the results of the study, successful outcome of the procedure was found in 17 cases (77%) of the SR group and 24 successful cases (38%) in the NSR group. The results of this study indicate that there is a significant difference in the reduction successful rate between the SR and NSR groups. The relative risk (RR) was 2.028 (p value = 0.002, 95% CI: 1.376 – 2.990) (Table II).

DISCUSSION

Intussusception is a common aetiology of acute abdominal conditions in paediatric patients. The worldwide average annual incidence ranges from 0.24 to 2.4 cases per 1000 live births, with an approximate male-to-female ratio of 2:1, with a significant occurrence in boys. Paroxysmal stomach discomfort, vomiting, red currant jelly stools and a palpable abdominal mass are among the classical symptoms and warning indicators.[6] In this study, we observed vomiting, red currant jelly stools and abdominal distension were in 21 (95.5%), 12 patients (54.5%) and eight (36.4%) patients in the SR group respectively. In the NSR group, vomiting, red currant jelly stools and abdominal distension were observed in 50 (79.4%), 39 patients (61.9%) and 27 (42.9%) patients. The clinical manifestation of intussusception varies between patients. Only 20% of cases have the classical clinical presentation, which includes stomach pain, vomiting, bloody stools and an abdominal mass. In addition, most children do not exhibit the whole symptom triad of abdominal pain, vomiting and bloody stools. Vomiting, irritability, fatigue, or bloody stools tend to be more prevalent among younger children, while older children commonly present with stomach pain. The clinical manifestation of intussusception aligns with the patient's age. It is imperative to underscore that these various characteristics, when observed, should heighten clinical suspicion for intussusception to ensure that infants with nonspecific symptoms receive a proper diagnosis.

Infant intestinal obstruction is frequently caused by ileocolic intussusception in order to avert the grave complications of intestinal necrosis, perforation, peritonitis, shock and potential fatality, it is imperative to swiftly and efficiently address intussusception. The established approach for managing intussusception is pneumatic reduction of intussusception (PRI) with fluoroscopic guidance, which entails the introduction of a catheter into the colon and inflation to a pressure range of approximately 80 to 100 mm Hg. Other therapeutic choice is hydrostatic reduction with normal saline or water-soluble contrast. Despite the fact that there aren't any studies that have examined discomfort during reduction yet, deep sedation is frequently used during colonoscopies, which is analogous. Recent investigations have shown the efficacy of reduction when anaesthesiologists provide the sedation. However, in most institutions, reduction is carried out on awake children without sedation.[9]

In this study we found that there are significant reduction successful rate differences between SR procedure and NSR procedure. The percentage of successful reduction rate in SR group is higher than NSR (RR 2.028 (95% CI 1,376 – 2.990; p = 0.002). The findings of this investigation align with those of Doo and Kim (2020), who reported a 65.1% success rate in

the performance of SR in 43 patients over a 3-year period. The achievement of successful reduction can be reliably facilitated by employing the SR procedure in conjunction with intravenous administration of ketamine, midazolam and atropine. This approach holds promise for reducing the necessity for surgical interventions in cases of paediatric intussusception.[10]

The findings of this study are consistent with previous study[12]. In their study, they conducted a total of 38 reductions, involving 31 patients and seven cases of recurrence. These reductions were performed using water under ultrasound guidance with sedation, resulting in a success rate of 76%. Importantly, no noteworthy adverse effects were documented in patients who underwent ultrasound-guided hydrostatic reduction under sedation. Notably, the success rate was notably higher in this particular group (p = 0.20). Factors that appeared to correlate with the need for surgical intervention included a greater length of the intussusception (p = 0.03), a location outside the right colon (p = 0.002), and a longer duration between the onset of symptoms and diagnostic imaging tests (p = 0.08). Poonai et al.[14] further supports the regular consideration of sedation in the case of children undergoing intussusception reduction. Importantly, it was established that the use of sedation did not exhibit any increased likelihood of adverse events (OR: 1.1; 95% CI: 0.6 2.1; p = 0.79) or perforation (OR: 2.1; 95% CI: 0.7 6.9; p = 0.21).[3]

The role of sedation in intussusception reduction remains a topic of ongoing investigation and debate. While certain studies have indicated lower success rates, others have reported a higher rate of achievement when sedation is employed. In our own clinical experience, we observed that in 10 to 14% of intussusception cases, which were initially unreducible by the radiology team and later assessed by the surgeon in the operating theatre after the administration of anaesthesia, the intussusception had resolved. This resolution was attributed to the beneficial effects of sedation, which promote muscle relaxation and reduce extraluminal abdominal pressure. These effects have been documented in prior research and are well-established in the field.[12] Fear and pain also can be reduced by using sedatives or anaesthetic during the enema treatment. Children have the ability to relax and work more cooperatively. A study found that applying midazolam to a small number of cases—just 16 in the atropine group and 16 in the control group increased the success rate of reduction from 68.8 to 93.8%.[8] Furthermore, a separate study demonstrated that deep sedation yielded comparable success rates to those achieved under general anaesthesia. The success of the reduction is also enhanced by employing the correct treatment and sedation regimen.[13]

In our investigation, the anaesthesiologist administered ketamine either as a standalone sedative or in conjunction with midazolam. Our study's findings find corroboration in the work of Shavit et al.,[16] who also employed the same sedation medication. The choice to utilise sedation based on ketamine appears suitable when considering the anaesthesiologist's familiarity with these drugs, the brief duration of the procedure, the necessity for immobilisation, the patient's age (over 3 months) and the discomfort associated with the procedure.[10,14] According to the guidelines of the National Institute for Health and Care Excellence regarding sedation for paediatric procedures, options such as nitric oxide, ketamine combined with midazolam or fentanyl are typically recommended. Midazolam is often combined with other sedatives to best suit the clinical requirements. Ketamine has a well-established track record for safety and efficacy in inducing dissociative sedation, leading to a trance-like and cataleptic state, offering substantial pain relief, sedation, immobility and amnesia. Although it can occasionally result in issues like laryngospasm, ketamine generally maintains airway reflexes, cardiovascular stability and spontaneous respiration. Particularly when used in conjunction with midazolam, it significantly reduces the incidence of vomiting, shortens induction time and enhances parental satisfaction when compared to using ketamine alone.[2,15,16]

Hydrostatic reduction is the preferred treatment for intussusception unless it's not advisable. The failure rate of hydrostatic reduction is greater when the mass extends beyond the splenic flexure. However, it's important to note that in most cases where hydrostatic reduction fails, they can be easily resolved through laparotomy.[17] In our study, the hydrostatic reduction procedure couldn't be successfully carried out in 44 patients from both groups. This indicates that medical professionals may not prevent the possibility of perforation since an ischemic or necrotic intestinal wall is more susceptible to such an event. Nevertheless, even if a perforation occurs during the reduction process, it has minimal implications for subsequent medical care because surgery ultimately becomes necessary in cases of unresolved intussusception. There is a lesser chance of problems can be realised with increased case finding and faster management.[7]

This study is offering valuable insights. However, its limitations include a small sample size, especially in the sedative group, which might affect the reliability of the findings. Also, the uneven distribution of cases between sedative and non-sedative arms could impact of interpretation the results. Despite these limitations, the study emphasises the importance of exploring sedation reduction techniques to improve procedures for children and minimise trauma. Further research is needed to fully understand their impact on paediatric outcomes and well-being.

CONCLUSION

The reduction successful rate of sedative procedure in hydrostatic reduction for children with intussusception is found to be higher than NSR procedure. Implementation of the SR procedure may reduce surgery rates in paediatric intussusception, thereby enhancing patient management. We contribute to evolve insight of non-operative approaches of paediatric intussusception management, particularly in Yogyakarta.

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ORIGINAL ARTICLE

The ameliorating potential of Citrus aurantifolia peel extract in the 2, 4, 6-trinitrobenzenesulfonic acid model of mice colitis

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ABSTRACT

Introduction: The number of inflammatory bowel diseases cases has increased throughout the years. Since, the current therapeutic methods have their adverse effects, this is leading to the development of alternative therapy derived from natural products.

Materials and Methods: In the present study, our objective was to explore the potential of Citrus aurantifolia peel extract (CAPE) on 2, 4, 6-trinitrobenzene sulfonic acid (TNBS) induced colitis in mice. Twenty-eight male Balb/c mice were divided into four groups: (1) normal group, (2) TNBS group, (3) 125 mg/kg CAPE group and (4) 250 mg/kg CAPE group. Colitis was induced through rectal administration of TNBS. The anti-inflammatory effects of CAPE against colitis were assessed by body weight, DAI score, colonic length, weight-to-length ratio, haematology profile and histopathological examinations.

Results: Our results showed that CAPE maintained the body weight of mice, repressed the increase of DAI score, maintained mice colonic length and weight, improved blood profile and suppressed the excessive production of TNF-α, IL-6 and IL-1β. Furthermore, CAPE improved the histopathological score of colitis mice.

Conclusion: All the findings of this study suggested that Citrus aurantifolia peel extract may be a potential natural agent for protecting mice against TNBS-induced colitis.

KEYWORDS:
Citrus aurantifolia peel extract, phenolic compound, colitis, TNBS, anti-inflammatory, mice

INTRODUCTION

Inflammatory bowel diseases (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), is a chronic remitting and relapsing inflammation that affects the gastrointestinal tract. The symptoms include acute abdominal pain, diarrhoea, mucosal inflammation of the colon and rectum, tissue damage, bloody stools, mucosa and pus.[1] Though the cause of IBD remains unknown, it is believed that IBD is associated with genetic and environmental abnormalities that cause the production of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6, leading to uncontrolled mucosa inflammation.[2,3]

According to the epidemiological study from Asia, the Asia-Pacific Crohn's and Colitis Epidemiologic Study (ACCESS), IBD incidence has increased in number specifically in Asian countries including Indonesia. From 2011 to 2013, the corresponding incidence rate was 1.50 per 100,000 of IBD, and the number was predicted to grow over the year.[4] Current medications for IBD have been palliative therapies, such as anti-inflammatory drugs, immunosuppressants and corticosteroids. However, functional food has currently become a healthy alternative to maintain the disease progression and it is derived from natural products. Epidemiological evidence shows that regular consumption of functional foods, value-added food products, and nutraceuticals is associated with a lowered risk of some chronic disease progression, including ulcerative colitis.[5]

Prior studies reported that natural bioactive compounds, such as phenolic, have anti-inflammation and antioxidant properties.[6] *Citrus aurantifolia* is a medicinal plant native to Southeast Asia and often used in various traditional medicine. It is one of the bitter orange species which possesses higher flavonoid and phenolic content compared to other bitter orange species such as grapefruit and lemon.[7] The fruits are extremely rich in phenolic compounds, but the peel contains an abundance of bioactive compounds that demonstrated potent anti-inflammatory activities. Well-established assays have also demonstrated that *Citrus aurantifolia* peel acts as a very powerful antioxidant.[8] However, the effect of *Citrus aurantifolia* peel on colitis has yet to be reported.

The study aimed to investigate the ameliorating effect of *Citrus aurantifolia* peel extract (CAPE) in a colitis mice model. The experimental colitis was induced by TNBS prior to treatment with CAPE. The anti-inflammatory effect was evaluated by DAI scores, histopathological observations, and determination of inflammation markers, including IL-1β, TNF-α and IL-6.

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MATERIALS AND METHODS

Citrus Aurantifolia Peel Extraction

Citrus aurantifolia were obtained from Colombo Market, Yogyakarta. The fruit was fully ripened, with green peel and 4 to 5 cm in diameter. Peels were manually separated using knife. The peel was freeze-dried at -48°C for 36 hours until the moisture content reached 10% and ground to obtain a Citrus aurantifolia peel powder (CAPP). CAPP was extracted using ultrasound-assisted extraction using 70% ethanol with the ratio of 1:30 (w/v) to yield CAPE. The extract was then concentrated under vacuum condition using rotary evaporator at 110 mbar for 2 hours and then suspended in 5% Na-CMC.

In-vivo experimental design and colitis induction

This study was approved by the Integrated Research and Testing Laboratory (LPPT) of Universitas Gadjah Mada (certificate number 00006/04/LPPT/IV/2022) and was carried out in accordance with the guidelines for the care and use of laboratory animals. A total of 28 adult male BALB/c mice (initial weight 30–40 g) were acclimated for 7 days to adapt to a new environment in a communal cage, under a controlled condition of 22–25°C with RH of 60–65%, AIN-93M feed and water available ad libitum. After acclimated, 28 mice were randomly divided into four groups with seven mice each. The groups were as follow: normal group (I), TNBS group (II), low dose CAPE group (III) and high dose CAPE group (IV). CAPE suspension in 0.5% Na-CMC was given at a volume of 10 ml/kg body weight. The CAPE dosage was determined according to the conversion of hesperidin dietary intake for human, resulting in 125 mg CAPE/kg body weight for low dosage and 250 mg CAPE/kg body weight for high dosage.[9,10] The normal group and TNBS group received water solution while CAPE groups received CAPE suspension once every day from day 1 to 7 days. The mice have then fasted for 24 hours after CAPE gavage.

On day 8, 100 mg/kg TNBS (Sigma Aldrich, USA) was used to induce acute colitis through enema. Mice were anesthetised using diethyl ether and a catheter (polyethylene, 1 mm diameter fixed on 1 ml syringe) was inserted into the anus about 3.5 cm deep, before 100 µl TNBS was injected into the colon. After the catheter was pulled out, mice were positioned vertically for 2 min. Throughout colitis period, all groups received the same treatment as pretreatment. Body weight, diarrhoea incidence, bloody stool and food intake were recorded daily. On day 18, mice were all sacrificed, and the colon was excised for further analysis.

Disease Activity Index

The severity of colitis was assessed daily in the 9 days after the induction of colitis using the disease activity index (DAI). DAI score was calculated as the sum of the damage scores for the criteria body weight loss, stool consistency, and faecal bleeding, in accordance with Liu et al.[11] The weight loss following induction of colitis was calculated relative to the initial weight of each animal prior to induction.

Blood Sampling Collection

Blood was collected through sinus orbitalis according to method by Parasuraman et al.[12] A capillary tube was inserted into the medical canthus of the eye (30° angle to the nose). Slight thumb pressure was applied to puncture the plexus/sinus so the blood will come through the capillary tube. 5 mL blood was then collected in heparin tube to be analysed immediately for haematology profile using haematology analyser KX-21 (Sysmex Corporation, Kobe, Japan).

Tissue collection and Biochemical Analysis

The colonic tissues were cut and then homogenised in phosphate buffered solution (1:9 [w/v]). The homogenised samples were subjected to centrifugation (5000 g) for 5 minutes. The cytokine levels of TNF-α, IL-6 and IL-1β were determined using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (FineTest).[13]

Histological Analysis

The colonic tissues were embedded in paraffin, cut at 5 mm, then mounted on clean glass slides. After the slicing was deparaffinised and rehydrated, the tissue was dyed with haematoxylin and eosin (H&E). All colon specimens were examined with an Olympus CX23 microscope to evaluate the ameliorating effect of CAPE on TNBS-mice colonic tissues. Histological gradings were assessed based on the criteria as reported by Bonfiglio et al.[14]

Statistical Analysis

Statistical analysis was performed using IBM SPSS version 23. The statistical significance of any difference in each parameter among groups was evaluated by one-way analysis of variance (ANOVA). All data was presented as the means ± S.E.M (standard error of mean).

RESULTS

CAPE Ameliorated TNBS-induced Colitis in Mice

Compared to normal group, body weight loss and an increase in the DAI score were observed in the TNBS group right after the day of induction. However, administration of CAPE in low and high dose managed to maintain the mice body weight and attenuate the DAI score, making them significantly lower than the TNBS group (Figure 1A-B). Shortening of colon length and increasing weight to length ratio were also observed in the TNBS group, which were used as indirect indicators of the severity of TNBS-mice colitis (Figure 1C-D). Compared with the TNBS group, CAPE intake attenuated colon shortening and reduced colon weight to length ratio.

CAPE Suppresses Inflammation in TNBS-Induced Colitis Mice

The level of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1β) in the homogenate supernatant of colonic tissues was determined by ELISA. Compared to the normal group, TNBS group showed a significant increase in TNF-α, IL-6 and IL-1β levels. However, the expression of the pro-inflammatory cytokines in the CAPE groups were significantly decreased compared to that in the TNBS group although the IL-6 levels of low dose and high dose group did not show a significant difference (Figure 2A-C).

Table I: Haematology profile of all mice groups.

Parameter	Unit	Normal	TNBS	Low Dose	High Dose
White blood cells	$\times10^3/\mu l$	6.07 ± 0.73[a]	16.58 ± 4.83[a]	7.03 ± 1.93[a]	6.82 ± 1.01[a]
Red blood cells	$\times10^6/\mu l$	10.47 ± 0.22[a]	8.79 ± 0.39[c]	9.95 ± 0.30a[b]	9.29 ± 0.22b[c]
Haemoglobin	g/dl	15.15 ± 0.38[a]	12.25 ± 0.70[b]	14.73 ± 0.30[a]	14.40 ± 0.56[a]
Haematocrit	%	52.08 ± 1.58[a]	42.68 ± 2.34[b]	50.78 ± 0.79[a]	49.77 ± 1.74[a]
Mean corpuscular volume	fl	49.72 ± 0.88[a]	48.5 ± 0.54[a]	51.03 ± 0.86[ab]	53.50 ± 0.88[b]
Mean corpuscular haemoglobin	pg	14.50 ± 0.41[ab]	13.93 ± 0.30[a]	14.80 ± 0.32[ab]	15.50 ± 0.42[b]
Mean corpuscular haemoglobin concentration	g/dl	29.12 ± 0.61[a]	28.68 ± 0.47[a]	29.00 ± 0.34[a]	28.93 ± 0.45[a]
Platelet	$\times10^3/\mu l$	1218 ± 60.20[a]	1046 ± 149.78[a]	1137.5 ± 33.91[a]	1134.83 ± 4.47[a]
Lymphocytes	%	75.73 ± 15.41[ab]	24.97 ± 15.29[a]	90.47 ± 2.85[b]	46.00 ± 20.69[ab]
Neutrophils	%	24.27 ± 15.42[ab]	75.03 ± 15.29[b]	9.53 ± 20.69[a]	40.54 ± 20.69[ab]

Different subscripts notated significant difference. Data (means ± SEM) was analysed by ANOVA.

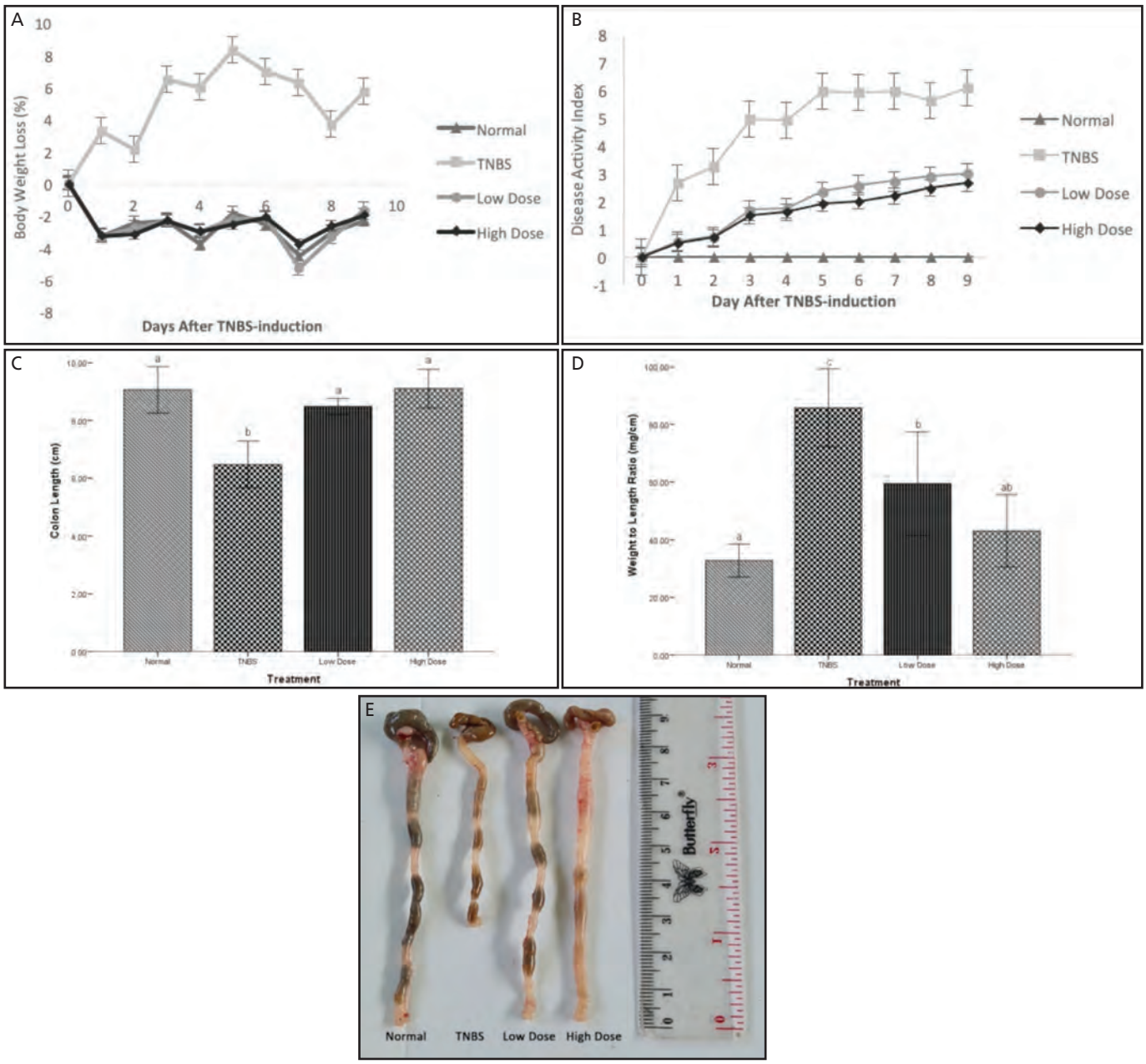

Fig. 1: Citrus aurantifolia peel extract (CAPE) ameliorates 2, 4, 6-trinitrobenzenesulfonic acid (TNBS)-induced colitis in mice. (A) Body weight loss compared to the previous day. (B) Change in DAI score. (C) Colon length of mice at day 9 after TNBS administration. (D) Weight to length ratio of mice colon tissues. (E) Representative pictures of colons. Normal: negative control; TNBS: positive control; low dose: 125 mg/kg CAPE; high dose: 250 mg/kg CAPE. Different scripts notated significant difference. Data (mean±SEM) was analysed by ANOVA

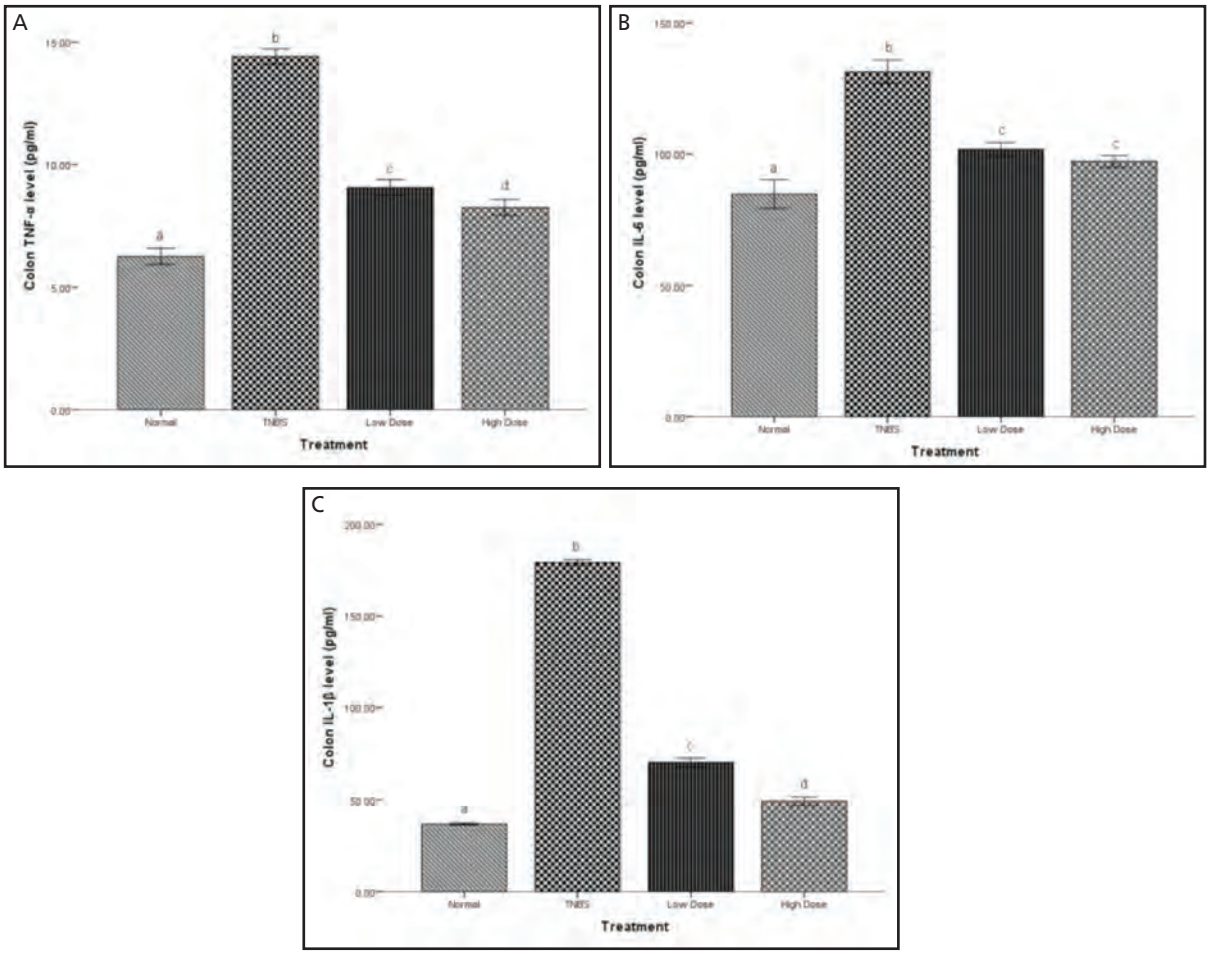

Fig. 2: CAPE suppressed the over-expression of pro-inflammatory cytokines (A) TNF-α, (B) IL-6 and (C) IL-1β in colonic tissues. Normal: Negative control; TNBS: Positive control; 125 mg/kg BW: 125 mg/kg BW CAPE; 250 mg/kg BW: 250 mg/kg BW CAPE. Different scripts notated significant difference. Data (mean±SEM) was analysed by ANOVA

CAPE Improves the Haematology Profile of TNBS-Induced Colitis Mice

An abnormality was observed in the blood profile of TNBS mice, as displayed in Table I. Compared to the normal group, TNBS group had higher white blood cells count and neutrophile while having lower absolute lymphocyte count and red blood cells count. The results emphasised the occurrence of inflammation in TNBS group. Administration of CAPE improved the blood profile of mice, as shown by the decrease of white blood cells count and neutrophile and the increase of red blood cells and absolute lymphocyte count. Therefore, it could be surmised that CAPE ameliorated TNBS-induced colitis mice indirectly through ameliorating their haematology profile.

CAPE Alleviates the Histological Damage in the Colon of TNBS-Induced Colitis Mice

Colitis was successfully induced in the TNBS group as the animals exhibited commonly found colitis symptoms, as shown by the decrease in body weight and an increase in DAI score. Compared to the normal group, the H&E-stained sections of colonic tissue of the TNBS group (Figure 3A-B) showed a serious inflammation with a scattered infiltration of monocytes, loss of histological structure and lesions throughout the mucosa. However, CAPE intake significantly reduced the inflammatory cell infiltration and morphological alteration, thus reducing the histological damage all over (Figure 3C-E).

DISCUSSION

IBD is a chronic remitting disorder of the gastrointestinal tract associated with mucosal inflammation that leads to the increased activation of adhesion, molecules and mucosal damage. While the exact cause of IBD is yet to be known, many studies have reported that oxidative stress is heavily involved in the exacerbation of colitis. Excessive pro-oxidant substances may cause mucosal injury and cause dysregulation of redox signalling that leads to an over-expression of pro-inflammatory cytokines.[15]

Over the past decade, functional food has emerged as a promising means to prevent and attenuate the disease progression of IBD. It affects the disease in various positive ways, for example, the consumption of quinoa, an edible grain-like crop, reduces the severity of histological damage in DSS-induced colitis mice due to the high amount of polysaccharide in quinoa thus promoting the growth of

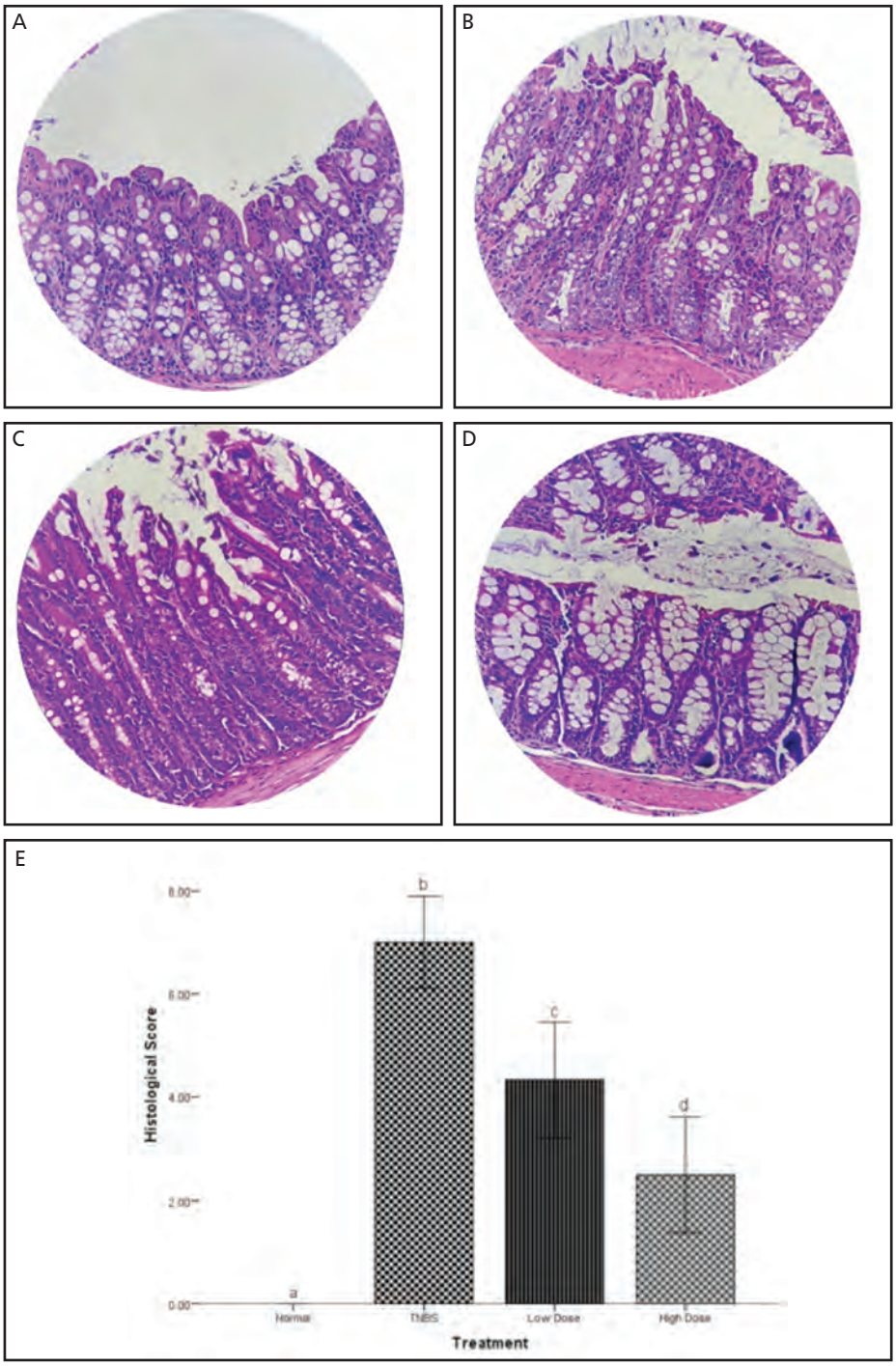

Fig. 3: CAPE attenuates histological injury in TNBS-induced colitis in mice. Representative images of haematoxylin and eosin (H&E) staining of colon tissue from (A) normal group, (B) TNBS group, (C) 125 mg/kg CAPE group, and (D) 250 mg/kg CAPE group. (E) Colonic histological score. Different scripts notated significant differences. Data (mean± SEM) was analysed by ANOVA

beneficial bacteria and the production of short chain fatty acids (SCFA).[16] Resveratrol, a polyphenol commonly found in grapes and peanuts, is reported to mitigate one of the main symptoms of IBD which is the disruption of the intestinal barrier in a Caco-2 cell model.[17] Several flavonoid compounds found in black ginger, namely, 3, 5, 7, 3′, 4′-pentamethoxyflavone and 5,7-dimethoxyflavone, also served the same effect.[18,19] In another case, the powder form of Citrus limon peel is able to repair the damage done to the colon tissue of DSS-induced mice and also alter the faecal SCFA composition which implies the change in intestinal microflora compositions.[20] On top of its various mechanisms, functional food is readily available in our daily lives, making it a more convenient choice as an alternative method to decrease the severity of IBD.

In this study, TNBS was chosen as a colitis inducer agent due to its significant similarities with human IBD. It is also one of

the most appropriate and successful models of experimental colitis. The pathology of TNBS-induced colitis is as such: transmural granulomatous inflammation associated with diarrhoea, rectal prolapse, weight loss and colonic wall thickening, all observed in human CD.[21] Infiltration of polymorphonuclear and mononuclear into the intestinal tissue, release of inflammatory cytokines (IL-1 and TNF-α) and an increase of inducible nitric oxide synthase (iNOS) and COX-2 were observed in TNBS-model of mice colitis.[22]

The over-expression of pro-inflammatory cytokines such as TNF-α, IL-6 and IL-1β is a common marker in IBD. It was induced by Toll-like receptor (TLR) ligands in monocytes, macrophages, T and B lymphocytes and is a sign of a compromised immune system. TNF-α is identified as the primary regulator of inflammatory responses involved in the pathogenesis of IBD.[23] Its over-expression result in a decrease in colonic mucosa layer thickness thus exposing colonic mucosa to luminal antigens.[24] IL-6 is another common marker in IBD, with its over-production heavily involved in dysregulation of immune responses and B-cell malignancies.[25] It is known to aggravate inflammation by directly inducing lymphocyte proliferation and differentiation through the nervous system.[26] The induction of TNBS acts as a potent oxidative agent that stimulates the T-cell, mimicking that of a human, thus causing an excessive expression of TNF-α, IL-6 and IL-1β.[27]

In our experiment, we administered CAPE orally before and during the colitis induction with TNBS and evaluated the therapeutic effects of CAPE. Induction of TNBS caused the loss of body weight, pathological changes of colonic tissue, and an increase in the tissue level of inflammatory mediators. However, daily oral gavage of CAPE protected BALB/c from various damages caused by TNBS-induction and ameliorated the body weight loss, DAI score, colonic weight and length, haematology profile, and histological scores. Moreover, the data showed that CAPE could decrease the production of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β in colonic tissues. The number of studies regarding the ameliorating effect of CAPE on colitis-induced inflammation is limited, but the results obtained are in accordance with studies regarding the anti-inflammatory properties of other citrus species. However, CAPE improved several parameters better compared to other citrus species peel extract, such as Citrus unshiu peel extract, which gave no improvement to the colon length of the treated DSS- induced mice and Citrus aurantium L. peel extract, which did not improve the DAI of the treated TNBS-induced mice.[28,29] Regardless, they were able to decrease the gene expression of inflammatory cytokines such as TNF-α, IL-6 and IL-1β, thus reducing their concentrations in colon tissue, which could also be seen in the treatment results using CAPE.

CAPE contained various bioactive compounds, one of them being phenolic. However, phenolic is a broad group. Therefore, it is difficult to identify the specific compound in CAPE that attributes to the attenuation of TNBS-induced colitis in mice. Previously, we studied the phenolic compounds in CAPE and found that CAPE contains high concentration of quercetin. Quercetin is a flavanol known for its anti-inflammation properties. It works as an anti-inflammation agent by blocking TNF-α-mediated inflammation by preventing TNF-α from directly activating extracellular signal-related kinase (ERK), c-Jun NH2-terminal kinase (JNK) and Nf-κB, which are potent inducers of inflammatory gene expression and protein secretion.[30] Quercetin may also indirectly prevent inflammation by increasing peroxisome proliferator-activated receptor c (PPAR-γ) activity, therefore antagonising Nf-κB or activator protein-1 (AP-1) transcriptional activation of inflammatory genes.[31] Other phenolic compound found in CAPE in an insignificant amount may also contribute to its anti-inflammatory properties thus making CAPE a remarkable anti-inflammation agent. Further identification is required, and the phenolic compounds contained in CAPE will need to be studied on their effects of TNBS-induced colitis in mice.

CONCLUSION

Our study showed that Citrus aurantifolia peel extract showed an ameliorating effect in TNBS-induced colitis mice. This effect is at least associated with its ability in maintaining relative balance between pro-inflammatory and anti-inflammatory cytokines namely the suppression of several pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6. Our results provide a new nutritional supplement perspective and a potential therapeutic remedy for attenuating the pathological conditions in IBD.

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Vitamin D ameliorates memory function in association with reducing senescence and upregulating neurotrophin mRNA expression in transient global cerebral ischaemic injury model in rats

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ABSTRACT

Introduction: Ischaemic stroke induces oxidative stress, mitochondrial damage, inflammation and senescence and the decrease of cognitive function. Vitamin D is a fat-soluble vitamin that has a neuroprotective effect to repair the function of the nervous system. The aim of this study is to investigate the effect of vitamin D on memory function, p16, p21 (senescence), and nerve growth factor (NGF) mRNA expression on the hippocampus after transient global cerebral ischemic.

Materials and Methods: The study was designed as quasi-experimental with a control group that only received post-tests. We performed in vivo study with an induction bilateral common carotid artery occlusion (BCCAO) model and vitamin D injection for 10 days. A total of 24 rats were divided into four groups (n = 6): Sham operation (SO [control]), BCCAO (transient global cerebral ischemic model not given vitamin D), VD1 (BCCAO + vitamin D 0.125 µg/kgBW), and VD2 (BCCAO + vitamin D 0.5 µg/kgBW). The spatial memory function was tested with the Morris water maze. We performed immunohistochemistry to localise p16 expression. p16, p21 and NGF mRNA expression were assessed by reverse transcriptase (RT-PCR) method.

Results: The vitamin D treatment group required shorter mileage to find the platform and probe test. The total time spent was longer in the target quadrant than in non-target. The Vitamin D-treated group had lower p16 and p21 mRNA expression and higher NGF mRNA expression than the BCCAO group. Immunostaining showed p16 signal in the pyramidal cell of CA1 area in the BCCAO group.

Conclusion: Vitamin D repairs memory function, senescence expression was lower and NGF was higher in the BCCAO model.

KEYWORDS:
Vitamin D, senescence, neurotrophin, memory, global cerebral ischaemic

INTRODUCTION

Stroke is a functional disorder of the brain caused by obstruction of blood flow to the brain due to bleeding (haemorrhagic stroke) or blockage (ischemic stroke).[1] The occlusion of the supplying artery blood to the brain affects a lack of adequate oxygen supply that disrupts cellular homeostasis which leads to death of nerve cells and impaired tissue function to a decrease in neurological function.[2] Cessation of brain tissue blood flow decreases the oxygen and glucose that are needed for ATP formation, resulting in a decrease Na+ K+ ATPase, which causes the membrane potential to decrease. K+ moves into space extracellular, while Na and Ca ions collect in the cell causing the cell surface to become more negative and triggering depolarisation of the membrane, resulting in structural changes in space and leading to tissue death in the brain.[2]

Ischemic injury-reperfusion results in disruption signalling of the oxidative stress response, which leads to mitochondrial damage, dysregulation of metabolic neuronal ca2+ homeostasis, dysfunction autophagy lysosome and proteasomes.[3] It also triggers activation of cellular senescence signalling that increases damage to mitochondrial structure and function, decreasing ATP production and disrupting normal cell metabolism.[4] Senescence incident will induce cessation of growth of cells competent in cell proliferation and differentiation. Senescence also triggers inflammation in neurons, loss of neuronal synapses and demyelination, resulting in cognitive impairment.[5]

Expression of cellular senescence can be characterised by the presence of changes in two major signalling pathways, namely cellular senescence markers p16 and p21.[6] Ischemic stroke research studies will accelerate the senescence-

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associated secretory phenotype (SASP). The transient middle cerebral artery occlusion (TMCAO) stroke model showed an increase in the expression of cellular senescence.[7] However, in the bilateral common carotid arteries occlusion (BCCAO) stroke model there are no research studies on increased expression of cellular senescence. Senescence in areas of the brain will impact on damage to the hippocampus, thereby disrupt factor signalling neurotrophins that play a role in cognitive function.[8,9]

Neurotrophin nerve growth factor (NGF) protein plays a role in synaptic and neuronal growth, myelination, differentiation and development neuronal. NGF is produced in hippocampus dentate gyrus, and pyramidal cells. NGF expression in the hippocampus is regulated by neural activity.[10,11] The hippocampus is a complex structure found in the temporal lobe which has important role in learning and memory. Previous research stated that an imbalance between reactive oxygen species, reactive nitrogen species and antioxidants triggers senescence cellular and decreased expression of NGF receptor in Alzheimer's animal model.[12,13] Furthermore, senescence triggers a decrease in nerve growth factors, resulting in a decline in cognitive function.[13]

Vitamin D is a fat-soluble vitamin that has the neuroprotective effect to repair the function of nervous system. Vitamin D has antioxidant properties that regulate cells differentiation, cells proliferation, peroxidation, neuroplasticity and axonal growth[14] moreover it plays a role in oxidative stress control. This study aims to elucidate vitamin D on spatial memory function, mRNA p16, mRNA p21 and mRNA NGF expression in a transient global cerebral ischemia model BCCAO.

MATERIALS AND METHODS

Study, Design, Location and Time

The study was designed as quasi-experimental with a control group that only received post-tests. The research was approved by Ethical Committee of Medical Research and Health of Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada with number KE/FK/1486/EC/2022. A total of 24 rats (Rattus norvegicus), 150 to 300 gm body weight (BW), were used. The rats were adapted to the cage conditions for 7 days with access to food and water ad libitium before the rats divided into four groups (n = 6): SO (sham operation), BCCAO (transient global cerebral ischaemic model not given vitamin D), VD1 (BCCAO + vitamin D 0.125 µg/kgBW) and VD2 (BCCAO + vitamin D 0.5 µg/kgBW).

Transient Global Cerebral Ischaemic Injury Model Bilateral Common Carotid Arteries Occlusion

A transient global cerebral ischemic model was created using BCCAO.[15] The rats were anesthetised using ketamine 100 mg/kg intramuscular. After that, the rats were positioned supine on the operating table, then incised at the anterior median line of the neck for exploration and visualisation of the common carotid artery. The right and left common carotid arteries were clamped with non-traumatic vascular clamp for 20 minutes. Next, the neck wall was sutured, and the rats were returned to the cages for recovery. In the SO group, the same surgical procedure was performed to visualise the bilateral common carotid artery without clamping the arteries.

Vitamin D Treatment

This study used calcitriol (1a, 25-dihydroxy vitamin D) (Cayman®) as the active vitamin D that was diluted in ethanol 0.2% until a concentration of 1 mg/ml is obtained. Vitamin D was given at different doses, namely 0.125 µg/kgBW in the VD1 group and 0.5 µg/kgBW in the VD2 group. Vitamin D treatment was done by intraperitoneal injection once per day for 10 days, with a volume 0.1 ml/100 grams BW. The SO and BCCAO groups were intraperitoneal injected with ethanol 0.2% in equal volume.

Assessment of spatial memory function using Morris water maze

The spatial memory function assessment instrument in this study used the Morris water maze (MWM). The device for the test consisted of a white-painted rounded pool (1.5 m in diameter and 0.4 m in height). The pool was divided into four virtual quadrants titled A, B, C and D. There was a rounded, white-painted platform (13 cm in diameter and 16.5 cm in height) located in the middle of a randomly chosen quadrant and kept in the same location throughout the experiment for each rat. Eight starting points were marked on the outside of the pool wall. Several coloured pictures were placed around the pool. These pictures served as distal clues for the rats to find the platform. The pool was filled with water and added with milk (1.5 to 2.5 cm above the platform) until the platform was barely visible. The assessment in the learning phase was to judge the total mileage to find platforms and test probes as the total time spent in the target and non-target quadrants in each treatment group. The first assessment was the learning phase which was done using a pointer location platform around the pool in the form of a picture on the wall of the pool called a distal cue. Platforms are first hidden by adding a mixture of water and milk. The pool was divided into eight quadrants and platforms are placed in one quadrant permanently. On evaluation learning phase, the experimental animal was placed at the starting point, swim towards the quadrant target and rise to the platform. Applications MWM would record time (sec) and total distance (mm) to find platforms at quadrant target. The learning phase was performed four times for four consecutive days. The probe test was performed 24 hours after the trial's final learning phase. The test was carried out once for 120 seconds without the use of a platform. The result obtained is the total time measurement spent on the target and non-target quadrants of each treatment group. Every test performed would record using a video camera with applications MWM.

Termination

The rats were terminated on the 10 days. Rats were anesthetised using 80 to 100 doses of ketamine mg/kg intramuscular then the abdominal wall is incised to open the abdominal and thoracic cavities. 0.9% NaCl solution into the ventricles cynically are used for organ perfusion. Decapitation was carried out to retrieve the brain tissue of the rats with the right cerebral hemisphere on a normal buffer formalin for paraffin embedded tissue process, while the hippocampus separated from the left hemisphere in RNA preservation solution, stored at 20oC for mRNA extraction.

Immunohistochemical Staining

3-µm thick paraffin sections were deparaffinised, next the antigen was retrieved using heat-induced antigen retrieval methods, followed by blocking peroxidase with 3% H2O2 in the PBS solution. Then, the slides were incubated with blocking serum and incubated with mouse 1st polyclonal antibody anti-p16 (Invitrogen MA5-17142; 1:100) overnight. On the following days, the slides were incubated with antibodies and diaminobenzidine (DAB). The results were captured under a light microscope (Olympus CX22®) through the OptiLab software with 400x magnification.

RNA Extraction and cDNA Synthesis

The hippocampus of rats was extracted according to the procedural technique described by the manufacturer of the Genezol RNA Solution (GENEzol™, Cat. No. GZR100). Then, 3000 ng of total RNA was used to synthesis the cDNA. The synthesis of cDNA was performed using the cDNA Synthesis Kit (SMOBio, RP1400) with PCR condition of 25°C for 10 minutes, 42°C for 50 minutes, and 85°C for 5 minutes.

Reverse Transcriptase Polymerase Chain Reaction and Electrophoresis

Reverse transcriptase-PCR (RT-PCR) was performed to examine the expression of following genes: p16 (forward: 5′-CGTACCCCGATACAGGTGATG-3′, reverse: 5′-ATACCGCCAAATACCGCACGA -3′), p21 (forward: 5′-GTGATATGTACCAGCCACAGG -3′, reverse: 5′-CAGACGTAGTTGCCCTCCAG -3′), NGF (forward: 5′-CGAAGGGGAGCGCATCG-3′, reverse: 5′ GACATTACGCTATGCACCTCAGA -3′), and GAPDH (forward: 5′-GTTACCAGGGCTGCCTTCTC-3′, reverse: 5′-TCCCGTTGATGACCAGCTTC-3′) was used as housekeeping gene. NFW and Taq Master Mix (GoTaq® Green Master Mix, Cat No. M7122) was used. The PCR was performed using the following condition: initial denaturation at 94oC for 2 min, the following steps mere repeated for 35 cycles (denaturation at 94oC for 10 s, annealing 51oC for 1 min to expression p16, 61.3oC for 1 min to expression p21 and 56oC for 1 min to expression NGF, continued extension 72°C for 1 min, and ended with cycles elongation 72°C for 10 min). RT-PCR products were analysed on 2% Agarose-gel with DNA ladder (Bioron, Germany, Cat No. 306009). Gene expression was quantified then with densitometry analysis with ImageJ® software and GAPDH was used to normalise expression. Results of electrophoresis were performed by ultraviolet light transillumination using Geldoc Syngene Gbox Chemi XRQ series.

Analysis of Statistics

Data were analysed using the SPSS 24 software, and the normality test was performed by using Shapiro–Wilk. Normally distributed data were analysed using One-way ANOVA. The significant $p < 0.05$, then proceeds with the analysis post-hoc multiple comparisons.

RESULTS

Vitamin D Ameliorated Spatial Memory Function Based on Learning Phase and Probe Test of MWM

The results showed that the BCCAO group distance effect on reached platforms was longer than the other groups (Figure 1A).[16] First day assessment showed all groups required a long total distance to reach platforms. The BCCAO group (1440.93 ± 315.73 mm; p = 0.004) required significantly longer distance to achieve platforms than the SO group (970.20 ± 134.83 mm). The VD1 group (1159.61 ± 206.86 mm) and VD2 group (1165.33 ± 286.61 mm) have total distance longer than the BCCAO group, but not significantly different. Assessment on the second day showed the total distance to reached platforms of the BCCAO group (1472.91 ± 372.89 mm) significantly longer compared to the SO group (1017.92 ± 289.35 mm; p = 0.036) and VD2 (718.20 ± 319.95 mm; p = 0.001). BCCAO group tend to be longer than the VD1 group (1140.91 ± 407.08 mm), but not statically different. The third day showed that the BCCAO group (1439.36 ± 431.20 mm) required significantly longer compared to the SO group (746.75 ± 324.27 mm; p= 0.001); VD1 (959.46 ± 277.59 mm; p = 0.015) and VD2 (730.67 ± 156.08 mm; p = 0.001). The fourth day of evaluation demonstrated that the BCCAO group (1341.61 ± 335.79 mm) required the total distance reach platforms to be significantly longer than the SO group (714.86 ± 300.27 mm; p = 0.001); VD1 (821.69 ± 336.09 mm; p = 0.006) and VD2 (688.17 ± 160.09 mm; p = 0.001). There was no difference between the VD1 and VD2 group on the third and fourth days.

The SO, VD1 and VD2 groups have hippocampus dependent-allocentric swimming strategy that showed swimming direct path the location platforms. In contrast, the BCCAO group has a hippocampus-independent egocentric strategy swimming that showed with thigmotaxis swim, which affected most of the time was spent swimming towards the pool wall and difficulty finding the platforms. The finding is correlated with the swimming track pattern learning phase in BCCAO groups compared to the SO, VD1 and VD2 groups (1B).[16] Next, Probe test were performed for assessment of the total time spent in the target quadrant (Qtarget) compared to the quadrant was not the location platform (Figure 1C).[16] Probe test based showed that the total time spent in the quadrants target group SO; Qtarget was significantly longer than the non-target quadrant (Q1,Q2 and Q3; p = 0.000). Total time spent in the target group quadrant BCCAO; Qtarget significantly shorter than the quadrant non target Q1 but the total time spent in quadrant the target group of BCCAO is significantly longer than the non-target quadrant (Q2 and Q3; p = 0.000). Moreover, the assessment of total time spent in the target group VD1 and VD2 quadrant target was significantly longer than in the quadrant non-target (Q1, Q2 and Q3 p = 0.000) (Figure 1C).

Vitamin D Downregulated p16 mRNA and p21 mRNA Expression as Cellular Senescence Markers

The results of p16 mRNA and p21 mRNA expression in all groups were normally distributed according to the Shapiro-Wilk test (p > 0.05). RT-PCR revealed significantly higher of mRNA expression of p16 and p21 in BCCAO groups compare to the SO group. This finding demonstrates that the BCCAO group upregulated cellular senescence marker of p16 and p21 mRNA expression. On the other hand, vitamin D treatment affected downregulation of p16 mRNA expression which was demonstrated by significantly lower mRNA expression p16 in VD1 group (0.20 ± 0.01; p = 0.006) and VD2 (0.16 ± 0.03; p = 0.000) than the BCCAO group as well as p21. Vitamin D

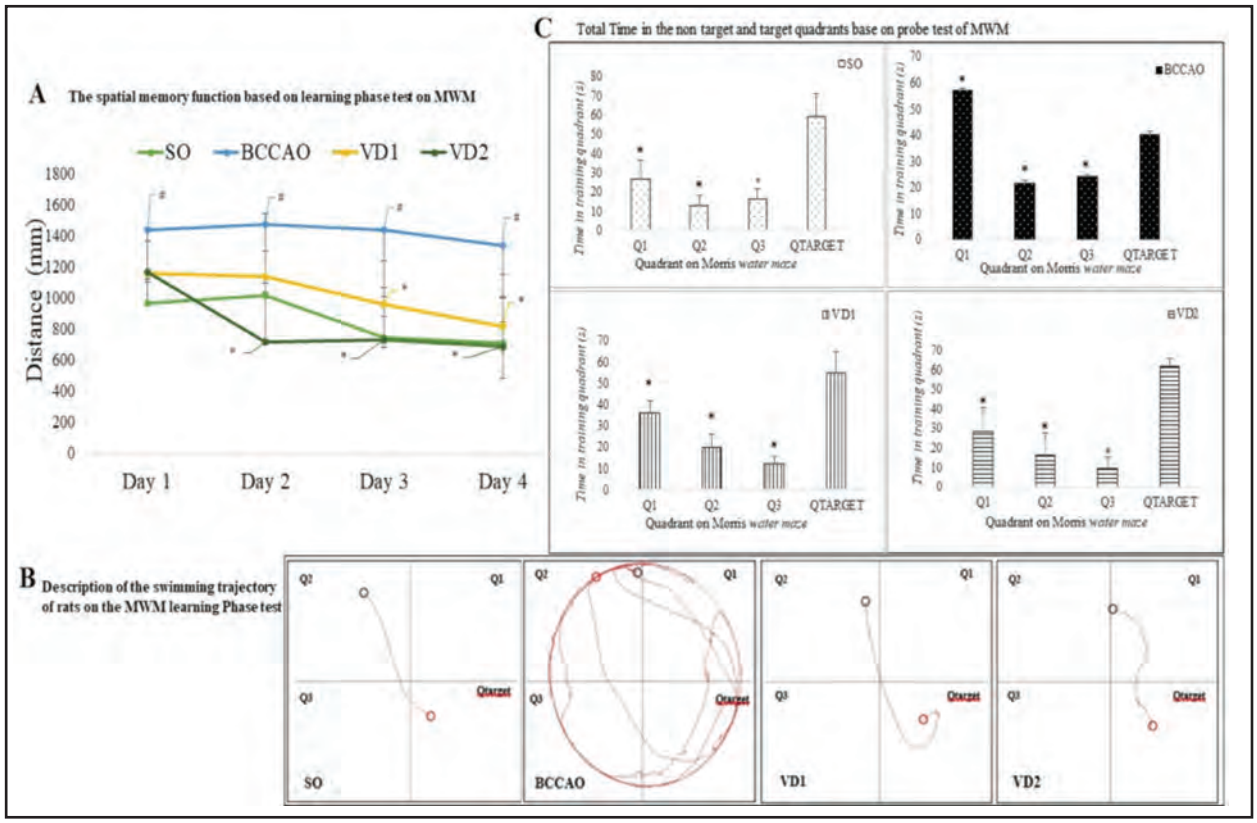

Fig. 1: Spatial memory function based on learning phase and probe test of MWM. (A) Mean ± SEM distance (mm) on learning phase of MWM by the group for 4 days. # $p < 0.05$ vs SO, * $p < 0.05$ vs BCCAO. (B) Swimming track pattern learning phase (total distance reached platforms on day 4). The black circle indicates the starting point, and the red circle indicates the endpoint swimming trajectory pattern based on the assessment learning phase of the SO, BCCAO, VD1 and VD2 rat groups. (C) The spatial memory probe test was assessed based on time spent in the target quadrant compared to the non-target quadrant. The data are presented in mean ± SEM. Probe test based on each group SO, BCCAO, VD1, VD2 quadrant target compared to quadrant non target (*$p < 0.05$).

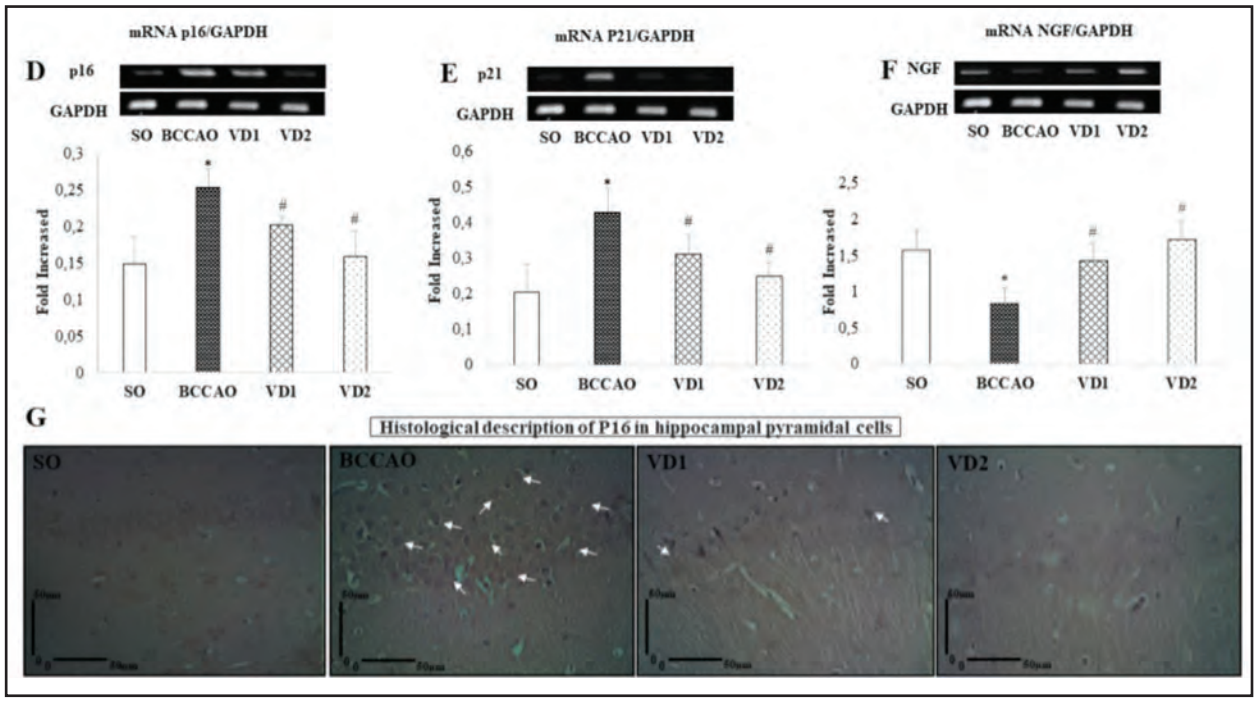

Fig. 2: (D) Representative image of electrophoresis results in RT-PCR products and mRNA expression of p16/GAPDH. (E) Representative image of electrophoresis results in RT-PCR products and mRNA expression of p21/GAPDH. (F) Representative image of electrophoresis results in RT-PCR products and mRNA expression of NGF/GAPDH. The data are presented in mean ± SEM. *$p < 0.05$ vs SO, # $p < 0.05$ vs BCCAO; (Analysed by one-way ANOVA, post-hoc LSD test). (G) Histological picture of the hippocampus. 400x magnification. (SO) Sham operation group; (BCCAO) BCCAO group; (VD1) BCCAO + Vitamin D 0.125µg group; (VD2) BCCAO + Vitamin D 0.5µg group. (White arrows: cellular senescence expression of p16).

administration showed p21 mRNA expression in VD1 group (0.33 ± 0.07; p = 0.024) and VD2 (0.25 ± 0.03; p = 0.000) significantly lower than the BCCAO group. Furthermore, p16 mRNA expression The VD1 group (0.20 ± 0.01; p = 0.006) was higher than the SO group. P21 mRNA expression the VD1 group (0.33 ± 0.01; p = 0.005) was higher than the SO group. In contrast, there are not significant difference between VD1 and VD2 groups (Figure 2D)[16] both in p16 and p21 mRNA expression (Figure 2E).[16]

Vitamin D Treatment Upregulated NGF mRNA Expression

The results of RT-PCR showed that NGF mRNA expression in the SO, BCCAO, VD1 and VD2 groups obtained data normally distributed based on the Shapiro-Wilk test (p > 0.05). NGF mRNA expression in the BCCAO group was significantly lower than the SO group (p = 0.000). mRNA expression in NGF group VD1 and VD2 were significantly higher than the BCCAO group (p = 0.000). The results of the treatment group VD1 with VD2 then VD1 and VD2 were compared with SO, but there was no significant difference (Figure 2F).[16]

Immunohistochemical Staining of p16 Protein Expression in Pyramidal cells of CA1 Area Hippocampus

p16 expression in the CA1 area of hippocampal pyramidal cells was observed by immunohistochemical staining. Immunopositive cells was indicated by colour browning of the pyramidal cells. In the BCCAO group, VD1, and VD2 have seen a brown colour is in the pyramidal cell CA1 area, showing that cellular senescence expression occurred in the hippocampus CA1 area. In contrast, in the SO group no brownish colour was seen in the CA1 area of hippocampal pyramidal cells (Figure 2G).[16] The cell membrane and cytoplasm are stained brown (severe sign white) is p16 expression, a marker of cellular senescence expression. The results of this observation indicated that global cerebral ischemic transient BCCAO model triggers the expression of cellular senescence at CA1 sites of hippocampal pyramidal cells.

DISCUSSION

This study revealed improvement in memory function in the Vitamin D treated group (Figure 1). MWM test results showed that memory function disruption in the BBCAO group occurred due to the induction of global cerebral ischaemic that led to damage the hippocampus CA1 area as an important region in processing learning and memory.[16] Transient global cerebral ischaemic BCCAO model affects damage to the hippocampus, resulting in cognitive dysfunction and significantly decreased function spatial memory.[15,16]

The BCCAO model caused a reduction of blood flow to most or all of the brain resulting in global ischaemic.[15] When blood flow is disrupted, oxidative stress (ROS) increases, affecting the expression of antioxidant enzymes[17] as the primary mechanism of nerve damage due to the transient global cerebral ischaemic.[18,19] Not only through the oxidative stress pathway but also inflammation response after ischemia, known as ischemic reperfusion injury (I/R injury), leads to neuronal damage effecting increase the expression toll-like receptors (TLR).[20] Increase in TLR results in an increase in factor activity transcription of NF-kB which is an inflammatory cytokine that is secreted when DNA damage occurs. NF-kB can directly activate p21 expression in response to DNA damage. The increased expression of p21 will trigger a senescence-associated SASP that will exacerbate the condition after ischemic stroke.[6,20,21]

The hippocampus is an important brain area for the formation, organization and storage of memories.[22] It is composed of pyramidal cells for projection of neurons spatial, contextual and emotional information.[22] Post-ischemia conditions lead to the direct changes in the synapse and vesicles of postsynaptic density that would affect the passage of impulse in the information delivery.[23,24] The increased of oxidative stress is a pathological disorder that triggers damage to the hippocampus. CA1 pyramidal cells in the hippocampus are one of the most vulnerable areas of damage due to transient global cerebral ischemic injury model, which decreases spatial memory function.[25,26]

The evaluation of the learning phase on the first day showed that all groups had trouble to finding the location platform. On the second day, BCCAO groups had difficulties continuing to find the platforms with the rats behaving in looking for wall contact on the edge of the pool and swimming in a manner thigmotaxis with its swimming strategy is the hippocampus-independent egocentric. In contrast, the vitamin D treatment groups showed the rat activity, which involved active swimming in locate behaviour platforms and swim direct-path reach soon platform with a swimming strategy in the form of hippocampus-dependent allocentric.[16,27,28] The evaluation of probe test in this study is based on the time spent in the target quadrant compared to the non-target quadrant. The analyses of probe test obtained that the vitamin D treatment group focused on the target quadrant, so the time spent in the target quadrant was longer than in a group of rats without vitamin D treatment (Figure 1C). Previous research by Moghaddasi's et al. using a cognitive function assessment (MWM test) showed that the cerebral hypoperfusion model damages the hippocampus of rat required a total mileage reach platform that is longer and time spent on fewer target quadrants than the control group.[29] Furthermore Curdt et al. research stated that a group of rats with damage to the hippocampus may experience memory loss and fail to form a strategy allocentric and finally depends on the strategy egocentric.[30]

An ischemic-reperfusion injury causes a degenerative state of cerebrum blood flow, followed by restoration of blood flow and oxygenation. Initial conditions of cerebral ischemia result in failure of pump Na+-K+ATPase, decreased tissue pH, depolarisation membrane, activation of Ca2+ channels causing an influx Ca2+ and releasing excitatory amino acids, especially glutamate, resulting in overactivation of glutamate receptors and increased intracellular Ca levels.[31] Reperfusion also results in tissue damage. Reperfusion will trigger increased oxidative stress and the production of excess reactive oxygen species (ROS) in the cerebral vessels. ROS leads to cell damage through DNA oxidation, peroxidation of cell membranes, and mitochondrial permeability transition pores (mPTPs) eventually triggering senescence activation cellular.[31,32] Continuous exposure to an injury can cause increasingly widespread cell senescence through activation of

the p16 and p21 pathways.[5] Cell senescence leads to progressive anatomical changes and atrophy also caused reduction of sensory perception in the central nervous system, then triggers further cell neurodegeneration and eventually results in decreased function cognitive.[5,6] Our study revealed that mRNA p16 and p21 expression in the BCCAO group was significantly higher than the SO group (Figure 2D and 2E). Similar result was also shown by Cheng et al. study that an increase in oxidative stress with manifestations of ROS production would trigger an increase in the expression of mRNA p16, mRNA p21 and mRNA p53 which was significantly higher than the control group.[32]

Neurotrophin factors are needed by neutrons for cellular processes, such as growth, neuronal differentiation, and cellular homeostasis in brain areas. Oxidative stress is one of the factors that decrease the production of neurotrophin.[33] In our study, the expression of NGF was lower in BCCAO group compared to the SO and VD groups. The BCCAO group experiencing stress or depression which significantly decrease the neurotrophin levels. Due to ischemic stroke, NGF is involved in development of brain structures, especially in the hippocampus and protected neurons. NGF can attenuate DNA damage, reduce ROS production and LDH levels and prevent apoptosis and neuronal senescence. Neurotrophin levels in older rats are significantly decreased compared to young rats.[16,33] Previous study examined the effect of stress on neurotrophin expression showed different results from this study that stress can increase neurotrophin expression (BDNF), possibly due to differences in stress-induced levels performed in experimental animal models.[34]

Vitamin D belongs to the group of fat-soluble vitamins metabolised by the body into an active form with various biological effects. The effectiveness of vitamin D has been widely published, including vitamin D as the regulator of calcium metabolism, hormone secretion, immune function, cell proliferation and differentiation processes, and as a membrane antioxidant that protects neurons against damage due to increased oxidative stress.[35,36] Effect of vitamin D administration on BDNF levels in young rats, middle age group and old age shows that vitamin D could increase BDNF levels by inhibiting oxidative stress signals.[36] Long-term therapy with calcitriol (1,25-dihydroxy-vitamin D) possibly inhibited the decreased density of hippocampal CA1 neurons due to aging cells by modulating the reduction of oxidative stress and inflammation. Vitamin D will induce protein synthesis which provides a neuroprotective effect against ROS-induced cytotoxicity.[37] Vitamin D induces SIRT protein, which plays a role in modulating the aging process, Klotho protein, which plays a role in anti-aging and Nrf-2 plays a role in factor inhibition NF-kB transcription resulting in decreased inflammation.[36,37,38]

The current research examines the effect of vitamin D on NGF in an experimental animal model of transient global cerebral ischemia, based on the results that NGF expression in the vitamin D treatment groups was higher than in the vitamin D untreated group. The results of this study are in accordance with the research Eriksdotter et al. reviewed the effect of vitamin D administration on NGF levels in rat hippocampus experimental animal models of Alzheimer's disease, NGF levels of the vitamin D given rat group were higher than those of the experimental animal model group not given vitamin D, high NGF levels will increase protection of neurons in the hippocampus.[12] The NGF levels increased in the hippocampus area will increase the growth of neurons, neurogenesis and functional recovery in a mouse model of stroke.[33,39] Spatial memory function test assessment in this study, the transient global ischemia BCCAO model given vitamin D 0.125 µg/kgBW (VD1) and 0.5 µg/kgBW (VD2), that the spatial memory function assessment was based on the learning phase and probe test, rats given vitamin D did better compared to the ischemic cerebral global transient model BCCAO group who didn't receive vitamin D. In this study, it was found that both doses of vitamin D had the same effect in attenuating senescence, increasing neurotrophins and were able to improve spatial memory function after transient global cerebral ischemia. This is aligned with the previous study which proved that both doses (0.125 µg/kgBW) also improve inflammation, and reduced epithelial cell apoptosis and fibroblast expansion in renal fibrosis.[40]

CONCLUSION

Vitamin D has neuroprotective effects on the hippocampus. Vitamin D improved memory function in a rat model of global cerebral ischaemic by attenuating cellular senescence and NGF, a cellular signal transduction that plays a role in the process of maintaining neuronal survival in the hippocampus.

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ORIGINAL ARTICLE

Risk factor for inappropriate use of prophylactic antibiotics in inguinal hernia repair surgery

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ABSTRACT

Introduction: There are some complications that can arise after surgery, like surgical site infection (SSI). In hernia repair, SSI incidence is low. Hence, the clinical practice guideline (CPG) published by the HerniaSurge Group (THG) does not recommend prophylactic antibiotics for hernia repair. Despite the unnecessary use of prophylactic antibiotics, regarding patient safety, prophylactic antibiotics can be used. However, each hospital has its own CPG and recommended antimicrobials based on the infection cases in its site. Regarding antimicrobial resistances, evaluating prophylactic antibiotics is essential to prevent increasing incidence of antimicrobial resistance cases. The aim of this study is to evaluate the use of prophylactic antibiotics in hernia inguinal cases.

Materials and Methods: This cross-sectional analytic study used patients' medical records between 2015 to 2020. Demographic data, surgery data and the used antimicrobial data were extracted and written in case report form. Identification of risk factors for inappropriate use of prophylactic antibiotics was done using logistic regression. Results: We identified 55 inappropriate times of preoperative prophylactic antibiotic therapy cases out of 80 cases and 63 cases in post-operative antibiotics were different from the guideline. Statistical analysis did not find any factor related to inappropriate therapy time.

Conclusion: The misuse of prophylactic antibiotics was frequently found regarding the duration of prophylactic antibiotics in both pre- and post-surgery setting. Nonetheless, no risk factor was identified with the inappropriate use of prophylactic antibiotics.

KEYWORDS:
Prophylactic antibiotic, hernia inguinal, gyssens

INTRODUCTION

Some post-surgery complications such as surgical site infection (SSI) may happen in a surgical procedure. In hernia repair, SSI incidence is low.[1–5] Hence, the clinical practice guideline (CPG) published by The HerniaSurge Group (THG)[1] does not recommend prophylactic antibiotics for hernia repair. Moreover, the inappropriate use of antibiotics increases the risk of *Clostridium difficile* infection, anaphylactic reaction and antimicrobial resistance (AMR).[1,6–8] Regarding patient safety, however, a prophylactic antibiotic may be used for a patient at risk and high-risk environment.[1,4]

To prevent antibiotic overuse and AMR, World Health Organisation in 2017 (WHO)9 classified antibiotics into assess, watch and reserve (AWaRe) based on probability of antibiotic getting resistance.[10] In 2019, WHO recommended that only antibiotics in assess class be used as prophylactic antibiotics.[10] Nevertheless, since every hospital's infection pattern and AMR cases are different, each hospital has its own CPG and recommended antimicrobials. Moreover, healthcare providers' knowledge about antimicrobials, especially a doctor, also influences the choice of antimicrobial drugs.[11] Hence, evaluation of antimicrobials prescription must be done. According to the Health Ministry of Republic Indonesia, Gyssen's flowchart can be used as a tool to evaluate antimicrobial prescriptions.[12]

Evaluating prophylactic antibiotics is essential to prevent the increasing incidence of antimicrobial resistance cases regarding AMR. This study aims to evaluate prophylactic antibiotics use in hernia repair surgery in our institute hospital based on its own CPG and identify risk factors for inappropriate use of prophylactic antibiotics.

MATERIALS AND METHODS

Study Design

This cross-sectional study used the medical record of inguinal hernia patients who underwent surgery between January 2015 and December 2020 with 18 years old as the minimum age. This study was approved by the Ethics Commission.

Immunosuppressed patients or patients in steroid therapy, pregnant or breastfeeding or at least have diabetes mellitus, heart diseases or chronic kidney diseases were excluded. A patient with positive bacterial culture 48 hours before surgery was also excluded. To minimise the bias during participant selection, any incomplete data was discussed further between authors.

Data Collection

Demography data such as age, sex, weight, height, comorbidities and length of stay were collected from patients'

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Table I: Modified Gyssens' Flowchart.[13]

A.	Appropriate decisions; all criteria of correct antimicrobial use are fulfilled
B.	Inappropriate indication; prescription of antimicrobials without the presence of infectious disease, or prescription of antimicrobials for an infection that does not need antimicrobial treatment.
C.	Inappropriate choice, including the inappropriate spectrum of the antimicrobial agent (too broad, too narrow, not effective), or inappropriate toxicity profile.
D.	Inappropriate application: inappropriate dosage, route of administration, administration time, and duration of therapy.
E.	Divergence from guidelines
F.	Insufficient data to judge the appropriateness of antimicrobial use.

Table II: Patients Characteristics.

Patients Characteristics	n
Total	80
Sex	
Male	79
Female	1
Age (years), Median (IQR)	62 (43 – 70)
18-29 years (%)	12 (15.00%)
30-39 years (%)	7 (8.75%)
40-49 years (%)	5 (6.25%)
50-59 years (%)	9 (11.25%)
60 years or more (%)	47 (58.75%)
Body Weight (kg), Median (IQR)	60.00 (54.5 – 68.5)
Body Height (cm), Mean (SD)	163.86 (±5.84)
Length of stay (day), Median (IQR)	2.45 (2.25 – 3.18)
Smoking Status	7 (8.75%)
ASA Status (%)	
1	17 (21.25%)
2	46 (57.5%)
3	17 (21.25%)
Wound Class (%)	
Clean	46 (57.5%)
Clean-contaminated	20 (25.00%)
Contaminated	0 (0.00%)
Dirty-infected	1 (1.25%)
N/D*	13 (16.25%)
Comorbidities	
Hypertension	30
Recurrent Inguinal Hernia	8

*N/D = No Data

Tabel III: Surgery profile.

Surgery Profile	n
Hernia Types (%)	
Reponibel	16 (20.00%)
Ireponibel/Incarcerated	13 (15.00%)
Strangulated	4 (5.00%)
N/D	48 (60.00%)
Surgery Cases (%)	
Elective	78 (97.50%)
Emergency	2 (2.50%)
Surgical Technique (%)	
Herniorrhaphy	72 (90.00%)
Hernioplasty	8 (10.00%)
Duration of surgery (Minutes), Mean (±SD)	51.52 (±16.01)

Table IV: The evaluation of the appropriateness of pre-surgery prophylactic antibiotics use.

Gyssens Criteria	n
Appropriate Use (%)	21 (26.25%)
Inappropriate Application (%)	
Wrong Doses	0 (0%)
Wrong Route of Administration	0 (0%)
Wrong Duration	0 (0%)
Wrong Timing	
Too Short	51 (63.75%)
Too Long	4 (5%)
*N/D	6 (7.5%)
Administration time before an incision (minute), Mean (SD)	121.18 (±74.87)
Divergence from Guideline (%)	0 (0%)
Insufficient Data (%)	13 (16.25%)

*N/D = No Data

Table V: Evaluation of the appropriateness of post-surgery prophylactic antibiotic use.

Gyssens Criteria	n
Appropriate Use (%)	0 (0.00%)
Inappropriate Application (%)	
Wrong Doses	0 (0.00%)
Wrong Route of Administration	0 (0.00%)
Wrong Duration	0 (0.00%)
Too Short	2 (2.50%)
Too Long	15 (18.75%)
Duration of antibiotic use after surgery (day), Median (IQR)	1.71 (1.57 – 2.49)
Wrong Timing	0 (0.00%)
Divergence from Guideline (%)	63 (78.75%)
Insufficient Data (%)	0 (0.00%)

Table VI: Univariate logistic regression for independent association with inappropriateness use of pre-surgery prophylactic antibiotics.

Variable	p value	OR (95% CI)
Age		
18-29 years	0.884	1.066 (0.455 – 2.497)
30-39 years	0.998	-
40-49 years	0.677	1.228 (0.467 – 3.229)
50-59 years	0.539	1.156 (0.728 – 1.836)
60 years or more	0.292	1.058 (0.953 – 1.174)
Length of Stay	0.920	1.041 (0.474 – 2.286)
Smoking Status	0.999	-

medical records. Types of hernia, hernia cases, surgical technique and duration of surgery were collected as surgery profiles. For prophylactic antibiotics evaluation, antibiotic drugs, dose, timing and route of administration were used based on the modified Gyssens' flowchart.

Statistical Analysis

Demography data was collected, analysed and reported as a percentage for all nominal data. Numerical data were reported in mean with standard deviation (SD) or median with interquartile range (IQR).

Appropriateness of prophylactic antibiotics prescription was analysed using modified Gyssens' flowchart[13] and reported in percentage. The modified Gyssens flowchart is shown in Table I.

To study the influence of demography data with appropriate prescribing, binary logistic regression was used while odds ratio (OR) and 95% confidence interval (95% CI) were given. Statistical significance was assigned at $p < 0.05$. All analyses were performed with IBM SPSS ver. 26.

RESULTS

Characteristics of Patients

A total of 84 patients were diagnosed with inguinal hernia and undergone hernia repair between 2015 and 2020. Four out of them were excluded because three were in steroid therapy and the one had an allergy to ketorolac and mefenamic acid. So, a total of 80 patients were included in this study. Table II shows the characteristics of the patients.

Surgery Profile
Reducible inguinal hernia dominated the cases with 16 out of 80 included data, followed by irreducible/incarcerated (13 cases) and strangulated (4 cases). The rest was missing. Table III shows the summary of the surgery profile.

Evaluation of Antibiotics Use
Ceftriaxone was used as a pre-surgery prophylactic antibiotic in all hernia repair patients with 2 g/day and can be divided into two doses. It was given intravenously.

Some errors were discovered during administration while using a modified version of Gyssens' flowchart with the hospital's CPG as a reference. Also, 13 data points were not included in the medical record. Table IV shows an overview of the evaluation of the appropriateness of pre-surgery prophylactic antibiotics use.

In post-surgery, same as pre-surgery, ceftriaxone was used, including doses and route of administration. Due to incomplete instruction in the hospital's CPG, CPG-THG was used as the reference for the post-surgery prophylactic antibiotic analysis. In CPG-THG, the duration of prophylactic antibiotics is limited to 5 days and only in incarcerated or strangulated cases. Table V summarises the evaluation of the appropriateness of post-surgery prophylactic antibiotics use.
After the patient was sent home, antibiotics were given. Cefixime with doses 2 × 100 mg doses is the most prescribed prolonged antibiotic (88.75%) followed by cefadroxil with doses 2 × 500 mg (11.25%).
Risk Factor of Inappropriateness Use of Prophylactic Antibiotic
Binary logistic regression did not show any significance between inappropriateness use of prophylactic antibiotics with demography data. The result is given in Table VI.

DISCUSSION

Inguinal hernia surgery at our institute hospital was performed using prophylactic antibiotics as recommended by the hospital's CPG. Despite the availability of CPG, inappropriate use of antibiotics was identified mostly in the pre-surgical medication. There is no information about antibiotics being used for post-surgical medication. Hence, the evaluation was based on CPG THG.[1] Unfortunately, the analysis did not find an appropriate use of post-surgery prophylactic antibiotics. Moreover, some cases used a prolonged antibiotics which is not recommended by CPG THG.

Risk factor analysis did not find any significance with inappropriateness use of prophylactic antibiotics. Nonetheless, Servesky et al.[14] reported that patients over 60 years were protective against SSI. Despite that, Servesky failed to track down the use of the prophylactic antibiotic. Hypothetically, there is any possibility that prophylactic antibiotics were used in these patients due to the immune system condition in geriatric can be considered, physiologically suppressed.[15]

Due to the inappropriateness in time of administration of pre-surgery prophylactic antibiotics, the hospital's CPG was reviewed. It was found that the time of antibiotic administration is not written well in the CPG either in pre-surgery or post-surgery setting. This may confuse healthcare workers about when to start and stop the antibiotics. In this study, all patients were given antibiotics from the first day since the patient was administered until discharge even at home. Meanwhile, prolonged antibiotics use does not give any benefit in any surgical cases (RR = 0.89; 95% CI = 0.79 – 1.00), except cardiac and maxillofacial surgery.[16] Hence, prophylactic antibiotics in hernia repair may be excessive. This misfortune should be noted as the excessive use of antibiotics increases AMR cases.[6,8]

Despite the inappropriate timing of pre-surgery prophylactic antibiotics, especially 51 cases of too long, this may happen perhaps because the antibiotics had already been given since the first day of patient stay in the hospital. Therefore, the antibiotic had been administered long before the incision. The 30 to 60 minutes of administration before an incision is referred by the American Society of Health-System Pharmacist (ASHP)[17] However, a systematic review by de Jonge et al.18 showed 120 minutes was the least tolerated time of prophylactic antibiotics administration. Otherwise, prophylactic antibiotics was not effective (OR = 5.26; 95% CI = 3.29 – 8.39). On the other side, the administration < 30 minutes also lost its protective property (OR = 1.07; 95% CI = 0.63 – 2.17).

In 2019, WHO recommends that only antibiotics in the assess group be used as prophylactic antibiotics while the rest must not be given unless multiple AMR is proven.[10] In our institute hospital, the recommended prophylactic antibiotic for hernia repair is ceftriaxone, while ceftriaxone itself has been listed in the watch group since 2017.[9] Regarding hernia repair, WHO suggests cefazolin, an assess antibiotic, as the prophylactic.[10]

While our institute hospital's CPG recommends using prophylactic antibiotics in hernia repair, CPG THG1 and the Ministry of Health of Republic Indonesia[19] are against it. For elective hernia repair, both guidelines do not support any use of prophylactic antibiotics due to a low incidence of SSI in hernia repair. Nevertheless, regarding patient safety, CPG THG recommends using prophylactic antibiotics limited to high-risk patients and high-risk environments.[1,20] A high-risk environment is defined as SSI incidences is higher than 5% in the control group in an observational study.[1,4] Serevesky et al[14] reported that inguinal hernia patients with diabetes mellitus, body mass index >35 kg/m^2, and smoking behaviour were at risk for developing SSI. Published by Cochrane library, a systematic review[4] revealed no beneficence of prophylactic antibiotics use for herniorrhaphy as it did not decrease SSI incidence (RR = 0.86; 95% CI = 0.56 – 1.33).[4] Meanwhile, prophylactic antibiotics could be considered in hernioplasty regardless the statistical analysis was not significant enough to show any benefit (RR = 0,78; 95%CI = 0.44 – 1.37).[4] The fact that there is a difference between our institute hospital's recommendation and up-to-date research article raises speculation that the inappropriate use of prophylactic antibiotics – either in pre-surgery or post-surgery – may often occur. Therefore, an update for hospital CPG is needed in order to reduce the inappropriate use of prophylactic antibiotics.

Despite the low incidence of SSI in hernia repair, the study highlights a significant misuse of prophylactic antibiotics both pre- and post-surgery. Therefore, clinicians should reconsider the routine use of prophylactic antibiotics in hernia inguinal cases and adhere more closely to evidence-based clinical practice guidelines. Meanwhile, the hospitals should regularly review and update their CPGs to reflect current antimicrobial resistance patterns and optimise patient outcomes.

The limitation of this study is that we only analysed the inappropriate use of antibiotics with demographic data of the patient and surgery profile at the time. The influence of physician's knowledge, attitude, and practice to choose either to use prophylactic antibiotic or not would give more comprehensive data, however, was not done yet in this study. Incompleteness of medical record was a challenged in this study. And the last, but not the least, because prophylactic antibiotic was used in all inguinal hernia patient since they had been admitted, no SSI were observed. Hence, the association between the use of prophylactic antibiotic and SSI could not be done.

CONCLUSION

The use of prophylactic antibiotics is debatable for preventing SSI. Hence, the evaluation regarding its use in clinical settings is important. In this study, the appropriate use of prophylactic antibiotics, both in pre-surgery and post-surgery settings, was evaluated. The misuse of prophylactic antibiotics was frequently found regarding the duration of prophylactic antibiotics in both pre- and post-surgery setting. Nonetheless, no risk factor was identified with the inappropriate use of prophylactic antibiotics.

CONFLICT OF INTEREST

The authors have declared there is no conflict of interest.

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Randomised post-test-only study of glutathione and ursodeoxycholic acid combination therapy on liver function in cholestasis-induced rats

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ABSTRACT

Introduction: Cholestasis is bile flow disruption that leads to bile accumulation, which could lead to liver fibrosis. Ursodeoxycholic acid (UDCA) has a hepatoprotective effect. Glutathione (GSH) is an endogenous antioxidant that plays a role in maintaining the function and structure of liver cells. This study aimed to examine the effect of UDCA-GSH combination therapy in multiple doses on liver function in the Sprague-Dawley rats' liver fibrosis model.

Materials and Methods: This was a randomised post-test-only study. A total of 28 rats were assigned into four groups: Group 1 is control group (C), samples had bile duct ligation and UDCA monotherapy 20 mg; Group 2, bile duct ligation + UDCA 10 mg + glutathione 10 mg (P1); Group 3, bile duct ligation + UDCA 20 mg + glutathione 15 mg (P2); Group 4, bile duct ligation + UDCA 30 mg + glutathione 20 mg (P3). Serum AST, ALT, ALP activity, total, direct and indirect bilirubin were collected. Shapiro-Wilk test was used for the normality test. All groups' data were compared using Kruskall-Wallis and Mann-Whitney tests.

Results: There was a significant difference in the ALP level in all rats and between the C and P2 groups. ALP level of all groups decreased significantly compared to the control group. Combination therapy group showed lower bilirubin levels. ALT levels significantly differed between the C-P1, P1-P2, and P1-P3 groups.

Conclusion: UDCA-GSH therapy improves liver function in BDL rats' models compared to UDCA monotherapy.

KEYWORDS:
Bile duct ligation, UDCA, glutathione, liver function

INTRODUCTION

Cholestasis is bile flow disruption that leads to the accumulation of bile in the blood, and the liver could lead to liver fibrosis. Pregnancy intrahepatic cholestasis, tumours, gallbladder stones, primary sclerosing cholangitis (PSC), and biliary atresia are the most common causes of cholestasis.[1,2] Hepatocyte tissue scarring is responded with cholangiocytes and hepatocytes by inducing the fibrosis process of periductal, biliary fibrosis, and liver cirrhosis.[3]

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are hepatocellular injury markers. ALT is a cytosolic enzyme that is found in high concentrations in the liver. ALT is usually higher than AST in most types of liver disease in which the activity of both enzymes is predominantly from the hepatocyte cytosol. Hepatocellular injury triggers the release of these enzymes into circulation.[4] Biochemical markers of cholestasis include elevated serum alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) levels.[5] These enzymes are located in the plasma membrane of hepatocytes. As bile acids accumulate in the liver, they act as detergents, releasing enzymes from the plasma membrane of hepatocytes.[5] Highly increased ALP, GGT, ALT and AST levels indicate obstruction of cholestatic liver disease.[6,7]

It has been hypothesised that oxidative stress may play a role in liver damage through various biological pathways. Bilirubin protects against oxidative stress by inhibiting the action of NADPH oxidase, which increases superoxide production. Moreover, bilirubin can quickly clear up peroxyl radicals, singlet oxygen, and hydroxyl radicals reactive nitrogen varieties[8,9] and minimise the alpha-tocopherol radical that promotes recycling in association with vitamin E.[10] In inclusion, bilirubin may have anti-inflammatory attribution and work as the significant anti fibrogenic agent through heme oxygenase-1 (HMOX1).[11]

Management of cholestasis involved ursodeoxycholic acid (UDCA), the lowest hepatotoxic profile among endogenous bile acids. UDCA has a hepatoprotective effect and pushes down the fibrotic rate of the liver. UDCA works through choleretic, immune system modulation, and cryoprotection mechanisms.[12] Its hydrophilic properties prevent hepatocyte damage due to bile acid accumulation.[13] In vitro study showed the hepatoprotective effect of UDCA in the amoxicillin-clavulanate hepatotoxic induced rat model.[14] However, UDCA lacks antioxidant properties, which is very important due to oxidative-stress induced fibrosis which is very common in cholestasis. Therefore, UDCA-antioxidant combination therapy needs to be studied.

Glutathione (GSH) is an endogenous antioxidant that plays a role in maintaining the function and structure of liver cells. Besides its antioxidant properties, GSH modulates cell growth

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Table I : Descriptive analysis and Kruskall-Wallis test

Variable	Group	Mean ± SD	Median (Min – Max)	p
ALP	C	1134.86 ± 71.43	1154 (1009 – 1211)	0.025*
	P1	925.14 ± 384.07	936 (337 – 1450)	
	P2	578.14 ± 194.20	449 (388 – 869)	
	P3	972.86 ± 313.34	1187 (573 – 1253)	
Total bilirubin	C	1.97 ± 2.48	0.5 (0.4 – 5.6)	0.077
	P1	1.18 ± 2.16	0.4 (0.3 – 6.1)	
	P2	2.14 ± 3.11	0.4 (0.2 – 6.8)	
	P3	0.33 ± 0.09	0.3 (0.2 – 0.5)	
Direct bilirubin	C	1.45 ± 2.08	0.3 (0.1 – 4.5)	0.309
	P1	0.82 ± 1.75	0.2 (0.1 – 4.8)	
	P2	1.48 ± 2.29	0.2 (0.1 – 4.9)	
	P3	0.15 ± 0.07	0.1 (0.1 – 0.3)	
AST (SGOT)	C	200.43 ± 68.65	159 (138 – 327)	0.919
	P1	173.71 ± 42.08	165 (134 – 265)	
	P2	219 ± 130.49	135 (106 – 405)	
	P3	168.71 ± 22.67	163 (140 – 206)	
ALT (SGPT)	C	110.57 ± 9.12	112 (95 – 122)	0.01*
	P1	80.43 ± 14.36	74 (71 - 111)	
	P2	122.43 ± 46.78	98 (82 – 193)	
	P3	114.14 ± 20.7	118 (93 – 144)	

Table II : Post-hoc Mann-Whitney test

Variable	Group	C	P1	P2	P3
ALP	C	-	0.209	0.001*	0.902
	P1	-	-	0.073	1.000
	P2	-	-	-	0.053
Total bilirubin	C	-	0.128	0.318	0.007*
	P1	-	-	1.000	0.259
	P2	-	-	-	0.383
Direct bilirubin	C	-	0.318	0.535	0.053
	P1	-	-	1.000	0.456
	P2	-	-	-	0.535
AST (SGOT)	C	-	0.805	0.620	0.710
	P1	-	-	0.710	0.902
	P2	-	-	-	0.710
ALT (SGPT)	C	-	0.004*	0.535	0.902
	P1	-	-	0.011*	0.004*
	P2	-	-	-	0.710

and death as inflammatory and hepatic fibrogenesis processes. The cholestatic patient has a low GSH level, affecting the likelihood of the fibrogenesis process.[15] UDCA-Glutathione combination therapy could increase hepato-protectivity against oxidative stress. GSH is easily found in nature and has been used several times, yet the effectiveness of UDCA-GSH combination therapy on liver fibrosis is unknown. Theoretically, GSH supplementation could increase the hepatoprotective effect on the liver, thus preventing liver fibrogenesis caused by cholestasis.[16]

UDCA-GSH combination therapy is superior to UDCA single therapy in reducing fibrosis in the liver fibrosis model Wistar rat. 20 mg oral UDCA and 15 mg intramuscular injection GSH were given in the previous study.[17] Therefore, this study aims to examine the effect of UDCA-GSH combination therapy in multiple doses on the degree of fibrosis in the Sprague-Dawley rats' liver fibrosis model.

MATERIALS AND METHODS

Experimental Animals

Male Sprague Dawley rats weighing 100 to - 200 g, aged 3 to - 6 weeks were housed at 28.0 ± 2.0 0C room temperature with 12 hour light/dark cycle and were fed rodent chow and water ad libitum. All animals were acclimated for 7 days before the experiment began. Medical Research and Ethics Committee Diponegoro University approved this study (protocol number: 32/EC/H/FK-UNDIP/IV/2022) and fully compliant with ARRIVE criteria.[18]

Fibrosis Model Rats' Induction

Cholestasis was induced by ligating the common bile duct. Before surgery, the rats were given 18 mg cefotaxime (Indofarma, Jakarta, Indonesia) via intramuscular injection as a prophylaxis antibiotic. Then, an intramuscular injection 0f 0,5 ml ketamine hydrochloride (Dexa Medica, Cikarang, Indonesia) was administered as anaesthesia.A midline laparotomy was performed under sterile conditions, and the rat's common bile duct was ligated with a 3-0 silk (DemeTECH, Miami Lakes, FL, USA). 7 mg oral Ibuprofen (Pharos, Semarang, Indonesia) was given every 8 hours/3 days to create pain-free experiment rats.

Animal Groups and Study Design

This study is a randomised post-test-only study with a control group. A total of 28 rats were randomly assigned into four groups (n = 7 per group) as follows: Group 1, samples had bile

duct ligation, and UDCA (Dexa Medica) monotherapy 20 mg, is control group (C). Group 2, bile duct ligation + UDCA 10 mg + glutathione (Sigma Aldrich, St. Louis, MO, USA) 10 mg combination therapy (P1). Group 3, bile duct ligation + UDCA 20 mg + glutathione 15 mg combination therapy (P2). Group 4, bile duct ligation + UDCA 30 mg + glutathione 20 mg combination therapy (P3). The dose of UDCA and glutathione was adjusted as pharmacokinetic of the drug for rats.[19]

UDCA was administered orally once daily, and glutathione was injected intramuscularly daily. All treatments were given continuously for 21 days.

Biochemical Analysis

Blood samples collected in centrifuge tubes were centrifuged at 3000 rpm for 10 minutes. The serum is stored at -20°C until it is used for biochemical assays. The appropriate kits were used to determine serum aminotransferase enzyme activities (AST and ALT) according to the calorimetric method. The ALP activity and total bilirubin (TB), direct bilirubin (DB) and indirect bilirubin (IB) were determined by colorimetric method.

Statistical Analysis

Results data were analysed using SPSS 27.0 for Mac Software. Data were expressed as a median. The Shapiro-Wilk test was used for the normality test. Then, all groups' data were compared using the Kruskall-Wallis and Mann-Whitney tests. All data were significant if $p < 0.05$.

RESULTS

Normality Test

The Saphiro-Wilk normality test revealed that most ALP, bilirubin, and aminotransferase level data did not have a normal distribution ($p < 0.05$).

ALP Level

Non-parametric Kruskal-Wallis test showed a significant difference in ALP level in all rats ($p = 0.025$). Mann-Whitney test showed a significant difference between the C and P2 groups ($p < 0.05$). We noticed a significant decrease in the ALP level of all groups compared to the control group. In this study, we can infer that glutathione combination therapy lowers the ALP level.

Bilirubin Level

We measure two levels of bilirubin: total bilirubin and direct bilirubin. This study shows no significant difference in bilirubin levels between experiment groups ($p = 0.077$). Further, the Mann-Whitney analysis revealed a substantial difference between the C and P3 groups regarding total bilirubin level. Other comparisons in these two parameters, total and direct bilirubin, did not show significance. However, the all-glutathione combination therapy group (P1-P3) showed lower bilirubin levels. Furthermore, the P3 group showed the lowest direct (0.15 ± 0.07) and total bilirubin levels (0.33 ± 0.09).

Aminotransferase Level

We quantitatively measured two liver function markers, AST and ALT. Kruskal-Wallis test showed a significant difference between all groups in the ALT variable ($p = 0.01$), while in the AST variable, the difference was not statistically significant ($p = 0.919$). The Mann-Whitney test result showed that ALT levels significantly differed between the C-P1, P1-P2, and P1-P3 groups ($p < 0.05$). However, the results were various, we can observe that P3 has the lowest level of AST (168.71 ± 22.67), but P1 has the lowest level of ALT (80.43 ± 14.36).

DISCUSSION

The principal result of our study is the inverse relationship between liver enzymes and GSH supplementation. UDCA is widely used due to its cytoprotective mechanism to preserve liver integrity in cholestasis hepatopathies.[20] Two years of UDCA (600 mg/day) or vitamin E (800 IU/day) treatment effectively reduced liver dysfunction in Indian NAFLD patients.[21] However, the efficacy of UDCA remains controversial.[21,22] UDCA monotherapy could not alter the level ALT, AST, and bilirubin levels in liver fibrosis model infant rats.[23] The UDCA therapy lacks antioxidant properties which oxidative stress would prove to be a major problem in cholestatic liver.[13,15] Cholestasis produces oxidative stress in the liver, as increased malondialdehyde (MDA) content shows. BDL rats also demonstrated they decreased water-soluble antioxidant potential and lipid peroxidation as reflected in superoxide dismutase (SOD), catalase, glutathione peroxidase (GTPx), and MDA level.[24] Oxidative stress contributes to hepatotoxicity induced by cholestatic liver disease.[25] Oxidative stress is the overproduction of highly active molecules, such as reactive oxygen species (ROS). The liver injury occurs when liver cells are exposed to certain noxious stimuli, leading to an imbalance between the oxidative and antioxidative systems.[26] ROS released by Kupffer cells (KCs) activate the hepatic stellate cells (HSCs), leading to an increase in the proliferation and synthesis of extracellular matrix (ECM), contributing to fibrosis and cirrhosis.[27] Oxidative stress (OS) associated with inflammation causes focal or zonal necrosis, hepatocyte destruction, and architectural disarray.[28]

GSH, consisting of L-cysteine, L-glutamic acid, and glycine, is currently the most studied antioxidant due to its involvement in oxidative stress, which interacts with and forms glutathione adducts during the protection against free radicals.[29–35] These effects seem essential in regulating cell proliferation and death by mediating the cell's main redox regulatory signalling pathway.[35,36] Previous studies have also shown that the supply of GSH prevents cell damage due to oxidative stress. In contrast, reduced glutathione levels contribute to the onset and progression of many diseases, such as liver fibrosis.[30,37] Under physiological conditions, the liver can resist oxidative stress through GSH synthesis in hepatocytes. In the present study, BDL rats treated with UDCA and UDCA-GSH exhibited low AST, ALT, ALP, and bilirubin levels, which indicated a reduction of oxidative stress and was accompanied by decreased tissue injury. GSH can directly scavenge radicals and peroxides via mixed disulfide formation or oxidisation to generate oxidised glutathione.[38–40] GSH can resist oxidative stress by serving as a

substrate for antioxidative enzymes, including GSH-Px, which converts hydroperoxide into less harmful fatty acids, water, and GSH disulfide.[40] Therefore, GSH can resist cholestasis-induced oxidative stress and attenuate liver fibrosis in advance.

Combination UDCA-GSH shows lower-level AST, ALT, bilirubin, and ALP in all graded doses compared to control (UDCA monotherapy), further alleviating liver fibrosis. In the present study, ALT and AST levels in the UDCA monotherapy group did not recover within the normal range, indicating that UDCA alone is insufficient for suppressing oxidative stress caused by cholestasis in BDL rats. Therefore, although UDCA-GSH treatment exerted a significant protective effect in BDL rats in the present study, hepatic oxidative stress continues. Limitations of the present study include the short duration of modelling and treatment and the fact that no healthy rats were present; therefore, results cannot represent the anti-hepatic fibrosis effects of UDCA-GSH compared to the baseline condition. A future study will likely clarify the therapeutic impact of UDCA-GSH on patients with cholestatic liver disease by improving the modelling method and experimental design and increasing the animal sample size. Future study with more complex variable such as liver biopsy will likely clarify the effects of UDCA-GSH.

CONCLUSION

This study demonstrates a favourable outcome of UDCA-GSH therapy on cholestasis in BDL rats' models compared to UDCA monotherapy by attenuating liver fibrosis based on liver function enzymes. Future study with more complex variable will likely clarify the effects of UDCA-GSH.

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ORIGINAL ARTICLE

Human dried amniontic membrane (H-DAM) as a biomaterial patch on gastric perforation wound healing: macroscopic evaluation

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ABSTRACT

Introduction: Gastric perforation is a rare occurrence, particularly in neonates. This is an emergency case in this population. The incidence of spontaneous gastric perforation in neonates is 1:2900 live births, with high mortality and morbidity rates. The primary treatment is surgical debridement and repair of the perforation, which has a high incidence of anastomotic leakage. At present, there is a plethora of studies investigating the efficacy of human dried amniotic membrane (H-DAM) technology in promoting wound healing. Consequently, researchers sought to ascertain whether there were differences in the number of adhesion and abscess classifications for the macroscopic evaluation of gastric perforation repair with H-DAM as a biomaterial in New Zealand white rabbits.

Material and Methods: A total of 30 male New Zealand rabbits underwent laparotomy and gastric perforation. These animals were then divided into three groups, with each group comprising 10 rabbits. Group 1 underwent primary repair, group 2 underwent omental patch repair, and group 3 underwent H-DAM patch repair. The rabbits were euthanised on the 7th day and the adhesion score and abscess classification were evaluated.

Result: A total of 30 samples of rabbits were homogeneous. On macroscopic evaluation, it was found that the H-DAM had the lowest mean adhesion score and the lowest incidence of abscess formation compared to all other groups.

Conclusions: It can be concluded that the utilisation of H-DAM as a biomaterial patch in the treatment of gastric perforation in the rabbit model did not result in any instances of leakage, adhesion or infection.

KEYWORDS:
Perforation, gastric, H-DAM

INTRODUCTION

Gastric perforation in neonates is an emergency case in neonatology that has a mortality rate. Gastric perforation in the neonatal period is a rare occurrence. Spontaneous gastric perforation in neonates has an incidence of 1: 2.900 live births, representing 15% of all cases of gastrointestinal tract perforation in neonates and children.[1] The three most common mechanisms causing gastric perforation in neonates are spontaneous perforation, ischaemia and trauma. Gastric perforation caused by iatrogenic trauma is due to the insertion of a nasogastric or orogastric tube. Such iatrogenic perforations are usually located along the major curvature and appear as puncture wounds or short scratches. Traumatic gastric perforation may also occur due to barotrauma during positive pressure ventilation. The ischaemic mechanism of perforation is difficult to elucidate as cases of perforation are associated with severe stressful conditions, such as prematurity, sepsis and neonatal asphyxia.[2,3] Surgery for gastric perforation involves debridement and primary repair. That method has a high incidence of leakage anastomosis in neonate with unstable condition or with poor systemic condition. Gastric perforation has high mortality rate 70%.[4,5]

Currently there are many studies on the use of human dried amniotic membrane (H-DAM) technology for wound healing. Amniotic membrane is the innermost layer of the three layers that make up the placenta. Amniotic membrane has antibacterial properties, low immunogenicity and can help the process of epithelialisation and wound healing.6 Therefore, researchers wanted to prove that there were differences in the number of adhesion and abscess score for the macroscopic evaluation in gastric perforation repair with H-DAM as a biomaterial in New Zealand white rabbits.

MATERIALS AND METHODS

A true experimental study was conducted on 30 samples of rabbits, with none of the subjects dropping out. The inclusion criteria for this study were as follows: New Zealand white rabbit males, aged 6 to 9 months, with a weight of 2 to 3 kg, deemed healthy and active. It should be noted that rabbits were excluded from the study if they had not fasted for a minimum of 12 hours. During the 12-hour fasting period, the subject exhibited aggressive behaviour, attacked other rabbits, and developed a surgical site infection. The data were analysed using the Statistical Package for the Social Sciences (SPSS) version 25.0 for Windows.

A total of 30 experimental rabbits were divided into three groups. Each group consists of 10 rabbits, the first group is primary repair group as control group (K), the second group was amniotic membrane group (A) and the last group was

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Table I: Adhesion score

	Criteria	Score points
Adhesion score	Are uterus, small intestine or omentum attached to the anastomosis? Is any other organ attached to the anastomosis? Feasibility of removing the adhesions bluntly with a swab	1 point per adherent organ 0 = no 1 = yes 0 = no adhesions in the first place 1 = all adhesions can be removed bluntly 2 = only part of the adhesions can be removed bluntly 3 = no adhesions can be removed bluntly at all

Table II: Abscess classification

	Classification	Criteria
Abscess classification	A	No macroscopically visible abscess formation (note That this does not preclude micro-abscesses visible in histopathological examination of the tissue)
	B	Limited, macroscopically visible abscess ("macro-abscess" for short)
	C	Disseminated peritonitis and/or free anastomotic leakage and/or intestinal perforation

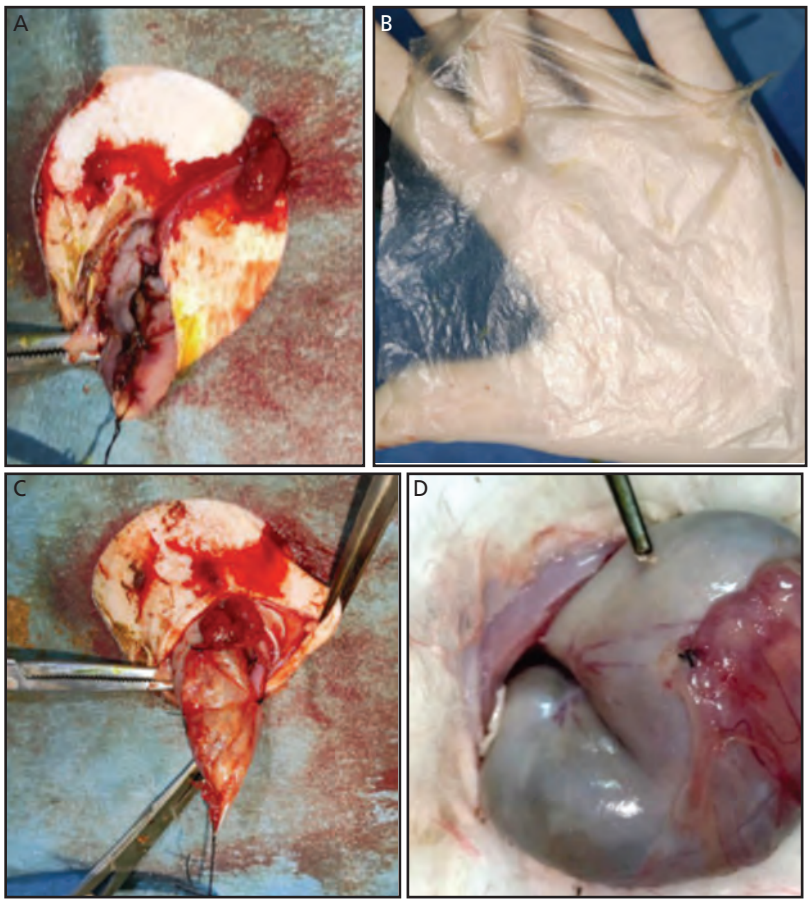

Fig. 1: A. Rupture in the gastric corpus was carried out by primary suturing, B. H-DAM measuring 10×10 cm, C. treatment group by placed H-DAM over primary sutures, D. treatment group by patching omentum over primary sutures.

omental patch group (O). In all three groups, a 2 cm long by 2 cm wide slice with a depth of the entire gastric wall was made in the gastric corpus and perforation repair was performed with 4 to 6 interrupted sutures using 4/0 non-absorbable silk multifilament thread. In group A, the repair was covered with a 4×2 cm wide H-DAM (H-DAM was sourced from the Tissue Bank at Dr. Soetomo Regional General Hospital in Surabaya, Indonesia. It was originated from human placental tissue that underwent a sterile drying process), with the basement membrane facing the serosa surface of the gastric corpus. H-DAM was fixed to the gastric serosa with 2 to 3 sutures. In group O, the repair was closed using the existing omentum in rabbits with the modified Graham Patch technique, namely omentopexy was

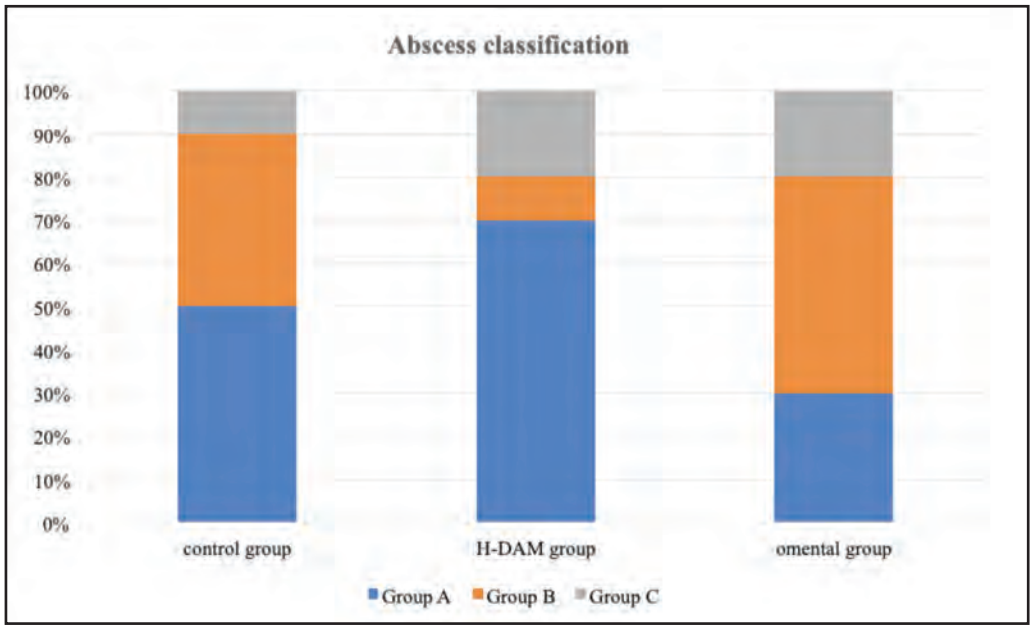

Fig. 2: Abscess classification of the control group : Group A had five animals, Group B had four animals, and Group C had one animal. Abscess classification of the H-DAM group: Group A had seven animals, Group B had one animal, and Group C had two animals. Abscess classification of the omental group: Group had three animals, Group B had five animals, and Group C had two animals.

performed by sewing the omentum facing the surface of the gastric serosa using silk 4/0 2 to 3 stitches. The abdominal muscle layer and skin are closed separately with silk 3/0 sutures in a straight line.

All samples will be terminated on postoperative day 7. Macroscopic evaluation was carried out by looking at the adhesion score and abscess classification on surgical site in each group based on the scores in Tables I and II. The data was then recorded and statistically analysed using SPSS 25 program.

RESULTS

A total of 30 samples met the research requirements, and no rabbits died during the study. The subjects were rabbits aged between 6 and 9 months, with a median age of 7 months in the control group, treatment group with H-DAM and treatment group using omental patches. The Lavene test based on age was employed to assess the homogeneity of the data. The resulting Sig value (0.934) exceeded the 0.05 threshold, indicating that the data were homogeneous. All research rabbits were male (100%), thus ensuring homogeneous data with respect to gender.

All samples were evaluated macroscopically on postoperative day 7 using a scoring system. The next step is to proceed with the one-way ANOVA test in order to analyse the mean adhesion score in each group. The statistical analysis employed non-parametric tests, notably the Kruskal-Wallis test, to assess the mean adhesion score across groups. The Asymp. Sig. 0.415 (>0.05) outcome indicates no significant difference in mean adhesion scores among the groups. The average adhesion scores are: control group (1.7), H-DAM group (1.4), and omental group (2.5). However, the group's mean adhesion scores reveal that the H-DAM group has the lowest score compared to all other groups

The H-DAM group showed the highest occurrence of group A abscesses (70%) compared to the control and omental groups, suggesting it had the least incidents of leakage and secondary infection. Group C (indicating peritonitis and/or anastomosis leakage) was present across all groups, with one in the control group, two in the H-DAM groups, and two in the omental groups, as depicted in Figures 2.

DISCUSSION

Macroscopic observations were conducted during surgical wound reopening in the H-DAM group shown in Figure 2. The H-DAM used for repair had fused with the gastric tissue without any adhesion with the surrounding tissue. The omentum remained unaffected, and there was no tissue leakage after repair.

In measuring abscess classification, seven (70%) rabbits in the H-DAM group did not form abscesses, with five (50%) in the control group and three (30%) in the omental group. This is due to the anti-inflammatory and antibacterial abilities of H-DAM, leading to a significantly lower incidence of anastomosis leakage and abscess formation when compared to the control and omental groups.

Gastric repair is characterised by multiple systemic, local and operative factors that collectively influence the continuum of wound healing. Intraperitoneal infection disrupts the wound healing process by prolonging the inflammatory phase and causing increased expression of tissue proteases.[8]

The antibacterial properties of amniotic membrane are made possible by elements such as interferon, lysozyme, transferrin, progesterone, immunoglobulins and B1c/Bla globulins present in the H-DAM layer. Dried amniotic membrane also reduces exudate by adhering tightly to the

wound. Dried amniotic membrane is also anti-inflammatory due to its abundance of compounds that inhibit inflammatory mediators such as proteases. Dried amniotic membrane contains high molecular weight collagen and growth factors that can facilitate epithelial cell proliferation, induce epithelial differentiation and prevent cell apoptosis. The anti-inflammatory action of H-DAM suppresses pro-inflammatory cytokines IL-1α and IL-β and produces natural metalloprotease (MMP) inhibitors. Its effects actively suppress T lymphocyte proliferation and inhibit monocyte differentiation. The amniotic membrane has natural inhibitors in its matrix that are able to stabilise matrix metalloproteinase expression in inflammatory environments, which plays an important role in the healing process.[8-11]

One of the challenges in this study is the postoperative care stage due to the numerous factors that can affect wound healing. A reduction in appetite after surgery can adversely affect wound healing in some rabbits, so it is vital to quarantine them before and after surgery to achieve the best possible results.

CONCLUSION

The use of H-DAM as a biomaterial patch for gastric perforation is expected to improve the healing process of gastric perforation compared to primary repair of gastric rupture without H-DAM. We conclude that the use of H-DAM as a biomaterial patch on gastric perforation in the rabbit model did not show leakage, adhesion and infection.

Further observations are necessary, especially under microscopic examination, to determine the collagen density, the number of fibroblasts, and other factors that affect the healing process, such as neovascularisation and inflammatory mediators, in repairing gastric perforation using H-DAM as a biologic dressing compared to omental patches in rabbit models. The researchers hope that this study will serve as a reference for further research in humans, particularly in children and infants.

CONFLICT OF INTEREST

There is no competing interest regarding the manuscript.

ETHICAL CLEARANCE

Ethics approval was obtained from the Animal Care and Use Committee (ACUC), Universitas Airlangga, Surabaya, Indonesia, with number 2.KEH.143.09.2023 before the study was conducted.

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None.

AUTHOR CONTRIBUTIONS

All authors equally contribute to the study from the conceptual framework, data acquisition, and data analysis until reporting the study results through publication.

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ORIGINAL ARTICLE

Calcitriol attenuates inflammatory response in the lung of diabetes mellitus rat model

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ABSTRACT

Introduction: Inflammation caused by diabetes can damage multiple organs, including the lungs. Vitamin D (VD) has been shown to potentially reduce inflammation and boost the immune system. VD might play a role in diabetes' inflammatory response. This study aims to elucidate the evidence regarding the lung as the target organ for DM and the possible role of VD in preventing pulmonary damage progression in the diabetes rat model.

Material and Methods: Thirty Sprague Dawley rats (3-month-old, 200 to 300 gm) were randomly divided into six groups, namely control (C), 4 weeks diabetes mellitus (DM1), 8 weeks DM (DM2) and three DM1 groups (VD1, VD2, and VD3) who received Vitamin D doses of 0.125, 0.25 and 0.50 µg/kg BW, respectively. After 4 weeks, daily VD was administered intraperitoneally for 30 days. Lung tissues were taken for IL-6, MCP-1, NFKB and CD68 mRNA expression analysis and paraffin embedding. Immunohistochemical staining against CD68 and MCP-1 was conducted. Data were analysed using one-way ANOVA. $p < 0.05$ was considered statistically significant.

Results: DM2 group represented significantly higher IL6, MCP1, NFKB and CD68 mRNA expression than Control group ($p < 0.05$). Meanwhile, VD2 and VD3 groups revealed significantly lower mRNA expression of IL-6, MCP1, NFKB and CD68 than DM2 ($p < 0.05$). Immunostaining revealed the spreading of MCP1 protein expression in lung tissue along with macrophage infiltration in the DM2 group, which was reduced in the VD2 and the VD3 groups.

Conclusion: VD shows a protective effect on diabetes-induced lung damage by regulating inflammation factors.

KEYWORDS:
Vitamin D, calcitriol, lung, diabetes, inflammation

INTRODUCTION

Diabetes mellitus (DM) is a chronic systemic disorder associated with many complications in various organs through neurological, microvascular and macrovascular damage.[1] The complex alveolar-capillary network of the lungs may be affected through diabetic microvascular damage. However, there is lack of study regarding diabetes effects in lungs tissue.[2-4] DM is associated with architectural and functional damage of lungs tissue.[4-6]

DM leads to various pathological responses one of which begins with an inflammatory process involving inflammatory cells, cytokines, chemokines and signalling pathways such as receptors of advanced glycation end-products (RAGE), janus kinase/signal transducer and activator of transcription (JAK/STAT), NFKB. The condition leads to end organ injury and damage as final consequences on DM. Excessive inflammation response in the lungs leads in deleterious effects as impaired lung function.[2,3] Patients with diabetes has been reported in increased risk of having lung diseases such as asthma, chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPD), pulmonary hypertension, cancer as well as infections.[2] Effects of diabetes conditions in end organ injury may give insight into prevention of diabetic progression and reducing the implication of diabetes mellitus, especially in lung injury.

Many substances have beneficial effects in inflammation, such as Vitamin D (VD). VD has been shown to potentially reduce inflammation and boost the immune system. Several experimental studies have shown that VD has an antioxidant effect by inhibiting the production of free radicals and oxidative modification of other biomolecules.[7] VD plays a role in inhibiting the chemotactic effect of monocytes/macrophages in inflamed tissues.[8] Previous study demonstrated calcitriol 0.05 µg/mL/100 gm BW/day attenuated inflammation and fibrosis in chronic kidney diseases model in rats.[9] Focusing on effects of diabetes condition on end organ injury, especially lung, VD might play a role in diabetes' inflammatory response, especially in lung tissues. This study aims to elucidate the evidence regarding the lung as the target organ for DM and the possible role of VD in preventing pulmonary damage progression in the diabetes rat model.

MATERIALS AND METHODS

Study Design and Diabetes Mellitus Induction

This quasi-experimental study had been approved by the Medical and Health Research Ethics Committee (MHREC) of the Faculty of Medicine, Public Health, and Nursing,

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Table I: Average blood glucose levels pre and post vitamin D (VD) treatment

Groups	Pre-VD Mean ± SD	Post-VD Mean ± SD	p-value
Control	111.4 ± 8.02	111 ± 9.43	0.807
DM1	379.2 ± 88.73	556.2 ± 126.88	0.043*
DM2	420.2 ± 94.61	566.2 ± 96.85	0.071
VD1	478.25 ± 100.83	308.75 ± 81.06	0.005*
VD2	362.8 ± 58.67	301.3 ± 81.87	0.245
VD3	414.6 ± 55.16	292.58 ± 87.34	0.144

DM1 = 1 month diabetic group; DM2 = 2 months diabetic group; VD1 = DM2 group treated with VD 0.125 µg/kgBW; VD2 = DM2 group treated with VD 0.25 µg/kgBW; VD3 = DM2 group treated with VD 0.5 µg/kgBW. *p < 0.05 post VD vs. Pre VD.

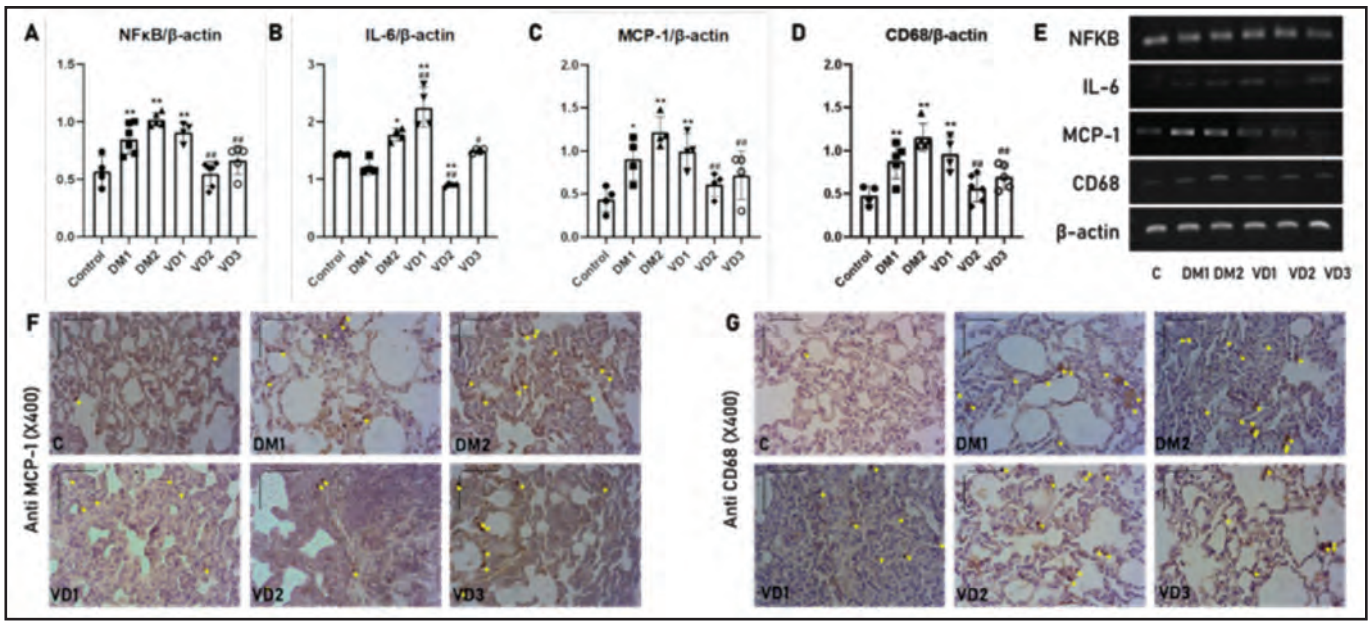

Fig. 1: (A D). NFKB, IL-6, MCP-1 and CD68 mRNA expression. *p < 0.05 vs control, **p < 0.01 vs control, #p < 0.05 vs DM2, ##p < 0.01 vs DM2. (E). Representative picture of anti-MCP-1 IHC staining with 400x magnification. The yellow triangle indicates the positive staining. (F). Representative picture of Anti-CD68 IHC staining with 400x magnification. The yellow triangle indicates the positive staining.

Universitas Gadjah Mada with the ethical clearance number KE/FK/1057/EC/2022. A total of 36 male Sprague Dawley rats (3-months-old, 200 to 300 gm) were randomly divided into six groups: control (C), DM 1 month (DM1), DM 2 months (DM2) and three variations of DM2 groups injected with several dosages of VD as the treatment groups. All rats were caged on a 12:12 hour's light-dark cycle under controlled conditions and given free access to standard diet and tap water.

The DM was induced by single intraperitoneal (i.p.) injection of streptozotocin (STZ) (13104, Cayman chemical) 60 mg/kg body weight (BW) dissolved in 0.1 M citrate buffer pH 4.5. Blood glucose level was quantified on day 6 post induction from the tail vein (pre-VD). DM was defined if the blood glucose level (BGL) was higher than 250 mg/dL. Rats that received the same amount of single i.p. injection of the solvent were used as control group (C) and euthanised after 2 months.

Calcitriol Administration

Diabetic conditions was performed in the rats for 1 month, then the rats with diabetic condition (blood glucose > 250 mg/dL) were randomised, and treated with Calcitriol in a month. After 1 month of DM induction, i.p. injection of VD in the form of calcitriol (71820, Cayman Chemical) dissolved in 1 ml of 0.2% ethanol were given for the treatment groups. The i.p. injection was administered once daily between 10 to 12 a.m. for 30 consecutive days. Three variations of the treatment groups based on the previous study (9) were VD1 (DM2 group treated with VD 0.125 µg/100 gm BW), VD2 (DM2 group treated with VD 0.25 µg/100 gm BW), and VD3 (DM2 group treated with VD 0.05 µg/mL/100 gm BW) per day. Higher dose was similar with our reference dose based on our previous study.9 The DM2 group also received the same amount of solvent during the same period.

Termination

Prior to euthanasia, the BGL of all rats was quantified (Post-VD). The rats in the DM1 group (after 6 weeks) and all other groups (after 8 weeks) were sacrificed by i.p. ketamine

injection (KTM100, PT. Bernofarm Pharmaceutical, Indonesia) at a dose of 100 mg/kgBW. After deep anaesthesia, the abdomen and thorax were then opened, and the left ventricle was perfused with phosphate buffer saline to clear the red blood cells from the lungs. The proximal bronchus of right lung was tied tightly and then the right lung lobes were carefully removed distal from the knot. A 22-G angiocath was then inserted and tied into lung trachea, through which neutral buffered formalin (NBF) was instilled with the pressure of 25 cmH2O. Lungs were then removed and immediately stored in 300 µl of FavorPrep™ RNA stabilization solution (part number: FARSS 001, Favorgen®, Taiwan) at -80oC for RNA extraction and neutral buffered formalin for paraffin embedding.

RNA Extraction, cDNA Synthesis and Reverse Transcriptase PCR

RNA from the lung tissues was isolated using FavorPrep™ Tri-RNA Reagent (FATRR 001, Favorgen® Biotech Corporation) according to the manufacturer's protocol. After RNA concentrations were quantified using a nanodrop, the cDNA was synthesised using ExcelRT™ Reverse Transcription Kit II, 100 Rxn (part number: RP1400, Smobio Technology, Inc., Taiwan). cDNA samples were then stored at -20°C. The cDNA was used for mRNA expression quantification using RT-PCR. These following primers were used for the RT-PCR: NFKB (forward: GCCTGACACCAGCATTTGA; reverse: CAAACCAAACAGCCTCACG), IL-6 (forward: TCCTACCCCAACTTCCAATGCTC; reverse: TTGGATGGTCTTGGTCCTTAGCC), MCP-1 (forward: GCTGTAGTATTTGTCACCAAGCTC; reverse: ACAGAAGTGCTTGAGGTGGTT), CD68 (forward: TGTGTCCTTCCCACAAGCAG; reverse: AAGAGAAGCATGGCCCGAAG), and β-actin (forward: GCAGATGTGGATCAGCAAGC; reverse: GGTGTAAAACGCAGCTCAGTAA), followed by electrophoresis procedure. Gene expression was quantified with densitometry analysis using ImageJ® software and normalised by ß-actin.

Immunohistochemical (IHC Staining)

After deparaffinisation, rehydration and antigen retrieval, the sample slides were douse with 3% H2O2 in PBS for endogenous peroxidase inhibition and incubated in blocking solution using Mouse/Rabbit Probe HRP Labeling Kit with DAB Brown IHC kit (Catalog number: TAHC03D-100; BIOTnA®). The slides were then incubated at 4°C overnight with primary antibody overnight, including anti CD-68 (ab955, 1:100, Abcam) and anti MCP-1 (ab25124, 1:300, Abcam). Then, the slides were incubated with species specific secondary antibody provided by the IHC kit at room temperature for 1 hour, followed by incubation with a DAB working solution. Haematoxylin was then used for re-staining. Examination across the entire visual field of the lung tissue was done under a light microscope (at least 400 × magnifications). A descriptive analysis was performed by comparing representative images from all study groups.

Data Analysis

The data obtained were analysed using IBM SPSS Statistics for Windows, version 26. Each parameter was described as the mean value ± standard deviation (SD). The data distribution of numeric data was tested with the Shapiro-Wilk test. One-way analysis of variance (ANOVA) test and post hoc least significant difference (LSD) test was used. Paired T-test was used to compare blood glucose level before and after VD administration. The value of $p<0.05$ was considered statistically significant.

RESULTS

Blood Glucose Level

After injection of STZ, the BGL of diabetic rats in our study ranged from 252 mg/dl to 585 mg/dl which indicated a successful model. BGL were taken before VD treatment (pPre-VD) and after VD treatment (post-VD) (Table I). BGL before VD treatment showed significant difference between all the diabetic groups with control group ($p < 0.05$). All VD treated groups had significant lower glucose level compared with both DM groups ($p < 0.05$), but not as low as the control group.

Diabetic Rats Showed More Severe Inflammatory Response than Non-Diabetic Rats

To explore the different inflammatory response between groups, we performed RT-PCR for inflammatory response-related genes and IHC staining for protein (Figure 1). The overall gene expressions, as shown in NFKB, IL-6, MCP-1, and CD68 mRNA expressions, exhibited significantly higher inflammation in diabetic rats, especially in DM2 group ($p < 0.05$), than in the control group (Figure 1A E). This data is supported by higher protein expression of MCP-1 (Figure 1F) and CD68 (Figure 1G). The anti-MCP-1 staining of the lung alveolar epithelium and interstitial tissue and anti-CD68 staining revealed higher macrophage cell infiltration and ultimately inflammatory reaction in rats with hyperglycaemia.

Calcitriol Attenuated the Inflammatory Responses in Diabetic Rats

To assess the optimum dose of calcitriol to prevent the progression of inflammatory in diabetic rats, we gave daily i.p. injection of calcitriol in three different dosages. The VD treated groups showed lower mRNA expressions of NFKB, IL-6, MCP-1 and CD68 compared to DM2 group (Figure 1A E), especially in group VD2 ($p < 0.01$) and VD3 ($p < 0.05$). Changes of inflammatory response were also detected in IHC staining of anti-MCP-1 and anti-CD68 in the lung tissues (Figure 1F G). The results indicated a marked decrease of inflammation in the VD treated groups, especially in dose 0.25 µg/kgBW and 0.5 µg/kgBW.

DISCUSSION

This study demonstrated that calcitriol treatment in diabetic conditions may ameliorate lung tissue injury and reducing inflammation responses. After administration of STZ, all of the diabetes groups had BGL results of over 250 mg/dl. This indicates the success of the diabetes induction.[10] The DM2 groups that were treated with VD showed a decrease in the BGL after a month of daily i.p. injection, especially VD 1 group. VD is hypothesised to exhibit glycaemic control property.[11,12] Circulating active form of VD, calcitriol, directly affects pancreatic β-cell function by binding to the vitamin D

receptor (VDR) on its membrane. It regulates the flow of calcium through the membrane in the pancreatic β cells, increasing insulin secretion and preventing apoptosis of pancreatic β cells as not to exacerbate DM. It also regulates the flow of calcium on insulin target cells in peripheral tissues and preventing occurrence of insulin resistance, thereby reducing BGL.[13] This property might be beneficial especially in diabetes patients with deficient VD status.12 Therefore, VD deficiency is thought to be the risk factor for development of diabetes.[14,15]

For a long time, the clinical significance of the relationship between diabetes and lungs was not well understood. However, Schuyler et al in 1976 were among the first to report the debilitating effect of diabetes in lung function.[2,5] Hyperglycaemia has been shown to be associated with airway inflammation.[2] In hyperglycaemia state, advanced glycation end-products (AGE) are actively produced and accumulate in the circulation and various tissues such as endothelium, smooth muscle cells, cardiomyocytes, neural tissue, and mononuclear cells.[16] Receptor for advanced glycation end-products (RAGE) is highly expressed in the pulmonary endothelium, bronchial and vascular smooth muscle, as well as in alveolar macrophages.[2,17] AGE increases the expression of RAGEs. Upon recognition of AGE by RAGE, oxidative stress production was accelerated in cells and then causes an inflammatory response through activation of NF-κB. NF-κB induced secretion of various cytokines and growth factors such as IL-6, IL-1α, TNF-α.[16,18,19]

Our study revealed upregulation of inflammatory mediator and macrophage marker CD68 in lung in diabetic condition, which showed complication of diabetes in lung inflammation. This downstream pathway of inflammatory response also causes increased in chemokines such as monocyte chemoattractant protein-1 (MCP-1), which recruits monocytes to inflammation sites.[2,30] This chemokine has been shown to predict a poorer prognosis in diabetes patients with asthma.[21] The macrophage infiltration could be detected by increased in cluster of differentiation 68 (CD68) in lung tissues.[21] Thus, inflammatory signalling which involving NF-κB and its downstream such as IL-6 and MCP-1 play role in inflammatory responses in the lung after diabetic condition. This increase in inflammatory response could be observed in our non-treated diabetic groups which heighten along with duration of hyperglycaemia. In previous diabetic mice model, there are association between diabetes duration with kidney injury and MCP-1 expression in kidney and urine.[23]

Inflammatory response could exacerbate the tendency of thrombosis, leading to the development of arteriosclerosis in micro and macrovascular pathway of chronic hyperglycaemia complication.[15,24] In endothelial cells, the AGE-RAGE system triggers nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and accelerates oxidative stress which causes endothelial dysfunction.[25] Endothelial dysfunction also causes an increase in MCP-1 and macrophage infiltration which further exacerbates the inflammation response.[22]

Our study highlights the effect of Vitamin D in downregulating inflammatory mediators signalling, especially NF-κB, IL-6 and MCP-1. We observed significantly lower pro-inflammatory factors in VD treated groups, especially VD 2 and VD 3 to DM2 groups. Vitamin D is a known anti-oxidative and immunomodulator. VD could lower the inflammation cytokine transcription through NFκB and mitogen-activated protein kinase phosphatase-1 (MKP-1) pathway.[26] VD also lowered monocyte/macrophage chemotactic through increasing expression of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα) which hinder the phosphorylation of NFkB p65, p38 MAPK and Erk1/2 in human adipocyte.[8] This immunomodulator features of VD in various organs of diabetes rat model such as liver,[27] kidney,[28] gingival epithelium,[29] and also pancreas.[30] The mRNA expression of MCP-1 and CD68 is supported with the histological findings. We observed lower MCP-1 and CD68 positive staining in VD treated groups compared to DM2 group. These findings could indicate the protective effect of VD through attenuating the inflammatory response in the lung tissues from DM. Quantification of protein level for assessing inflammatory mediator using western blotting or ELISA may give better understanding for this study, and it's may become limitation of the study. Quantification of macrophage infiltration may be important also for the next study. This result also may give perspective in application of VD in diabetic condition, both in pre-clinical or clinical setting which may give better outcome. However some additional data for functional analysis and more comprehensive quantification from both transcriptomic and proteomic analysis are needed for better understanding.

CONCLUSION

This study demonstrated that VD may ameliorate inflammation in the lung in diabetic condition. Adding lung function assessment and elucidating the possible stress oxidative pathway and fibrosis as the end-result of chronic inflammatory reactions may provide a better understanding about the potency of VD to prevent diabetes complications in lungs tissue.

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Optimal early surgery timing for congenital diaphragmatic hernia: A systematic review

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ABSTRACT

Introduction: Congenital diaphragmatic hernia (CDH) is a failure of closure of the pleuro-peritoneal canal due to faulty embryogenesis caused herniation of intra-abdominal contents into the chest. There needs to be more clarity about the optimal surgical timing for CDH. The aim of this study is to determine the optimal surgical timing for CDH using a systematic review analysis.

Materials and Methods: Our study used the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) 2020. The literature search approach used publications between 2013 and 2023 using Pubmed and SagePub databases. Studies were included if they contained reports of the best timing for emergency surgery for CHD repair. We did not include review articles and unpublished data.

Results: Five articles met the criteria. The overall result, the first pre-operative 24-hour oxygenation index mean, was temporally reliable and representative (intraclass correlation coefficient = 0.70, 95% CI = 0.61–0.77). Within any severity level, there were no differences in 90-day survival or mortality rate between delayed repair and early repair (p = 0.002). As a result, there is no optimal timing for surgery in severe cases of CDH. A delay in repair did not predict an increased risk of death, nor did it suggest an increased need for post-operative extracorporeal membrane oxygen therapy.

Conclusion: Regardless of the severity of the illness, the timing of CDH repair does not affect the mortality rate.Surgery is done after the physiology index achievement.

KEYWORDS:
Congenital abnormality; congenital hernia diaphragmatic; emergency surgery

INTRODUCTION

Congenital diaphragmatic hernia (CDH) is herniation of intra-abdominal contents into the chest as a result of faulty embryogenesis and due to failure of closure of the pleuro-peritoneal canal. The right pleuro-peritoneal canal closes faster than the left in intrauterine, resulting in a left-side posterolateral defect (Bochdalek's hernia, 75%). The rest of CDH may occur on the right side, central defect, congenital absence of diaphragm or anterior (Morgagni hernia).[1,2] Embryopathy and mechanical compression factors from diaphragm muscle abnormalities cause the triad of pulmonary hypoplasia, pulmonary hypertension and cardiac dysfunction with overall survival of 50%.[3] Closure of the pleuroperitoneal tract has not yet occurred by the time the midgut returns to the abdomen during the 9th and 10th weeks of gestation.[4] This congenital anomaly occurs in 1 in 2400–5000 births, with a 2:1 male-to-female ratio and a 5:1 left-to-right diaphragm hernia ratio and 40–50% die. Permissive hypercapnia and delayed surgery repair improved survival rates to over 75%.[5,6] The cause of CDH is unknown, suggesting that CDH may be caused by exposure to genetic predisposition and susceptibility to environmental factors. The CDH impact on lung development and function has been well established.[7]

Pulmonary hypoplasia and reactive pulmonary hypertension are CDH newborns' respiratory problems. The most severely impacted neonates exhibited respiratory difficulty at delivery and respiratory symptoms within 24 hours. Asymmetrical scaphoid abdomen and bulging chest are characterised in newborns.[5,6] Some researchers wait until the infant has released mechanical ventilation and needs a lower ventilator setting before surgery. In contrast, others follow the severity of pulmonary hypertension with serial echocardiographic examinations and wait until it stabilises.[7] There are no practice or simple clinical indicators to determine the surgery's optimal time.

Infants predicted to have good lung development (good lung growth on an antenatal scan, left-sided defect and no liver involvement) should be considered for the non-intubation breathing trial. Early echocardiography shows the degree of cardiac and coexisting defects, pulmonary hypertension and the right-to-left shunt for a medical management guide.[8–12] This study aims to determine simple clinical criteria for the best time for emergency surgery in a CDH.

MATERIALS AND METHODS

Protocol

By following the Preferred Reporting Items for Systematic Review and Meta-Analysis (PRISMA) 2020 rules, these

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Table I: The literature included in this study

Author	Origin	Method	Sample Size	Result
Cox[13]	United States of America (USA)	Retrospective cohort study	158 neonates	OI readings are stabilised over time and change very little after 24 hours. When surgical repair of CDH is put off past the point of initial stability, the number of days on a ventilator and the age at which the patient is released goes up without any improvement.
Yamoto[14]	Japan	Prospective cohort study	276 patients with isolated left-sided CDH	The study indicates that surgery should not be performed within 24 hours of birth for patients whose CDH is of moderate severity, that there is no benefit to delaying surgery for more than 72 hours in patients whose CDH is of mild severity, and that there is no definitive optimal time to perform surgery in severe cases of CDH. These findings suggest no optimal time to perform surgery in severe cases of CDH.
Deeney[15]	United States of America (USA)	Retrospective cohort study	77 neonatal patients with Bochdalek hernias	Implementing pulmonary arterial pressure of 80% of systemic pressure in echocardiogram as a non-invasive method before CDH repair may reduce acute post-operative decompensation. There were no differences existed in longer-term survival.
Okuyama[16]	Japan	Prospective cohort study	477 neonates with isolated CDH	It does not appear that the timing of CDH repair impacts 90-day survival, regardless of the severity of the condition. Patients whose injuries are of a moderate degree may benefit from early repair because it shortens the duration of treatment.
Hollinger[17]	United States of America (USA)	Prospective cohort study	1,385 CDH Registry infants without pre-operative extracorporeal membrane oxygen therapy (ECMO)	When considering all known risk variables, the timing of CDH repair in low-risk newborns does not appear to affect mortality. Nevertheless, the clinical factors that guide the time of elective CDH repair continue to be unknown.

authors ensured it met the requirements. This protocol provides that the conclusions of the inquiry are accurate.

Criteria for Eligibility

In this literature review, we investigate the best timing for emergency surgery for CHD repair to demonstrate the relevance of the difficulties of the criteria of optimal early surgery.

The researchers needed to fulfil the following requirements: 1) The papers were written in English, and the best time for emergency surgery for CHD was determined. The published manuscript must meet both criteria. 2) The studies published after 2013 and before this systematic review are deemed relevant. The studies were not permitted to include editorials, did not have a DOI, published review articles and were identical to published papers.

Search Strategy

We used "congenital hernia diaphragmatic bochdalek"; "optimal timing of surgery"; "baby/neonate"; "neonatal/baby mortality" and "prediction factor" as keywords. The studies search in the systematic review was carried out from July, 11th2023 using the PubMed and SagePub databases by inputting the words: *"congenital OR "congenitally" AND "bochdalek" OR "bochdaleks" AND "hernia, diaphragmatic OR "hernia" AND "diaphragmatic" OR "diaphragmatic hernia" OR "diaphragmatic AND "hernia" AND "optimal" OR "optimality" OR "optimally" OR "optimisation" OR "optimisations" OR "optimise" OR "optimised" OR "optimiser" OR "optimisers" OR "optimises" OR "optimising" AND "timely" OR "timing" OR "timings" AND "surgery" OR "surgical procedures, operative" OR "surgical" AND "procedures" AND "operative" OR "operative surgical procedures" OR "general surgery" OR "general" AND OR "general surgery" OR "surgery s" OR "surgerys" OR "surgeries" AND "baby neonate" AND "neonatal baby" AND "mortality" OR "mortalities" OR "mortality" AND "predict" OR "predictabilities" OR "predictability" OR "predictable" OR "predictably" OR "predicted" OR "predicting" OR "prediction" OR "predictions" OR "predictive" OR "predictively" OR "predictiveness" OR "predictives" OR "predictivities" OR "predictivity" OR "predicts" AND "factor" OR "factor s" OR "factors" AND y_10 AND clinicaltrial* used in searching the literature.

Data Retrieval

After reading each study's abstract and title, the writers examined and determined whether the study satisfied the inclusion criteria. The writers decided which previous research was selected for the article source. This conclusion was drawn after looking at many studies with the same no. All submissions must be written in English and cannot be seen elsewhere.

Only those papers that satisfied all inclusion criteria were considered for the systematic review, reducing the number of results to only those pertinent to the search. We do not assume any study's conclusions that do not satisfy our requirements. After this, the research findings will be analysed in great detail. The following pieces of information

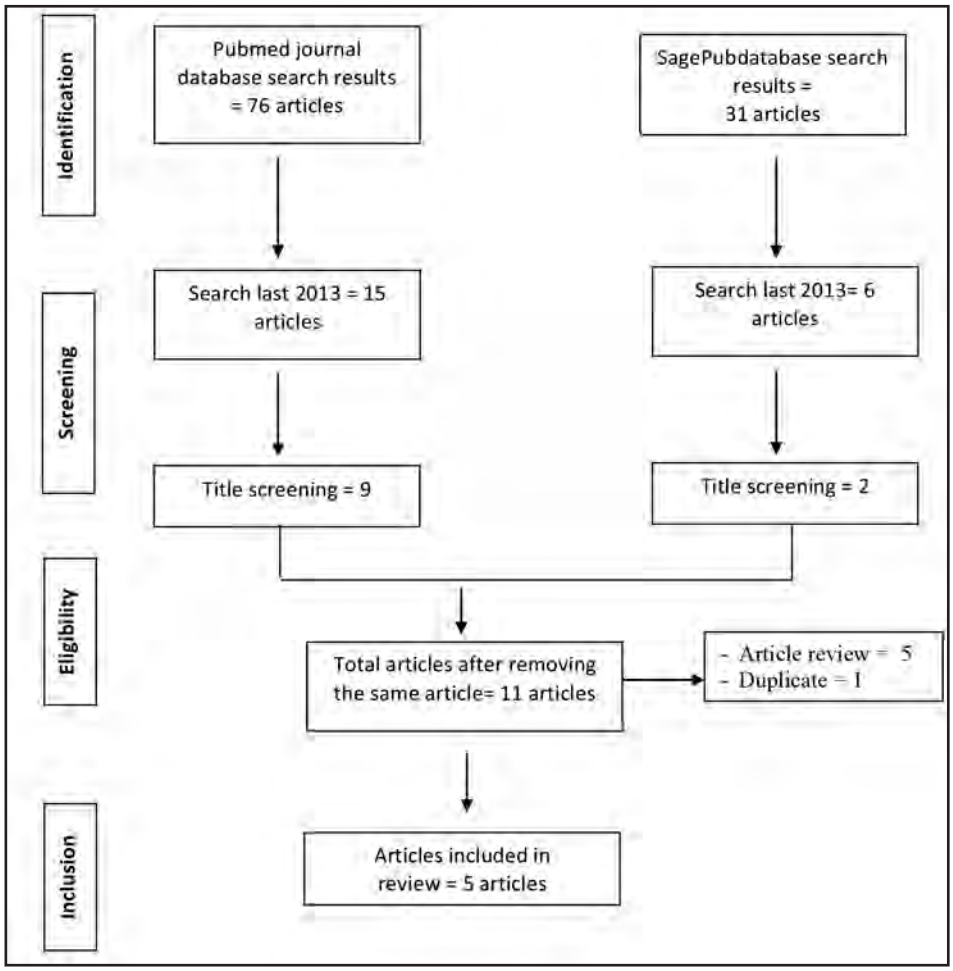

Fig. 1: Article search flowchart

were uncovered due to the inquiry that was carried out for this study: names, authors, publication dates, location, study activities and parameters.

Quality Assessment and Data Synthesis

Each author studied the research in the publication's title and abstract before deciding which journals to explore further. The next step will be to evaluate all of the articles suitable for inclusion in the review because they match the criteria set forth for that purpose. After that, we will determine which articles to include in the study depending on the findings that we have uncovered. These criteria are utilised in selecting papers for further assessment to simplify the process of selecting papers to evaluate. Which earlier investigations were carried out, and what elements of those studies made it appropriate to include them in the review are being discussed (Figure 1).

RESULTS

In the PubMed database, our search results included 76 articles, whereas on SagePub, 31 articles. The search results for the 2013 last year PubMed were 15 articles and six articles for SagePub. In the end, we compiled 11 papers, 9 of which came from PubMed and 2 from SagePub. We included five research that met the criteria.

Cox et al.[13] showed the first 24 h oxygenation index (OI) pre-operative mean was temporally reliable and representative (intraclass correlation coefficient (ICC) = 0.70, 95% CI = 0.61–0.77). A pre-operative OI of ≤ 9.4 (AUC = 0.95) predicted survival. An increased ventilator day (1.4, 95% CI = 1.1–1.9) and discharge age (1.5, 95% CI = 1.2–2.0) after the surgical delay in OI ≤ 9.4. When prospectively cohort, an OI ≤ 9.4 reflected pre-operative physiologic stability. OI stabilises by 48 h, and delayed repair (DR) beyond OI ≤ 9.4 may be associated with a more prolonged ventilator and hospital course without a survival benefit.

Yamoto et al.[14] repaired in 24–47 h had a decreased death rate compared to the other groups. There were no statistically significant variations in fatality rates between the four groups for moderate or severe cases. G2 dramatically enhanced survival rates in mild cases, compared to G1 (< 24 h), leading to a better overall outcome. Multivariate analyses showed that G2 had a lower mortality rate than the other groups. There were no significant differences in mortality across the four groups in mild and severe cases.

Deeney et al.[15] showed groups with comparable initial characteristics. In group 2 (repaired CHD after echocardiogram-estimated pulmonary artery pressure ≤80% systemic blood pressure specific criteria implementation), post-operative decompensation occurred less frequently than in group 1 (corrected after protocol implementation) (17% vs

48%, p<0.01). Similar results were obtained after adjusting for repair type, hepatic herniation and prematurity (15% vs 37%, p = 0.04). 94% of Group 2 patients survived 30 days after surgery, compared to 80% of Group 1 patients (p=0.06).

Okuyama et al.[16] showed that 90-day survival differed considerably between the three severity levels ("mild" = 97%, "moderate" = 89% and "severe" = 76%, p = 0.002), there were no variations in 90-day survival between DR and early repair (ER) within any severity level. There were no changes in treatment time between ER and DR in the "mild" condition. Treatment duration in the "moderate" category was shorter in the ER than in the DR (ventilation 11 vs 16 days, oxygen 15 vs 20 days and hospitalisation 34 vs 48 days). Treatment time in "severe" was shorter in ER than in DR, whereas the best OI was higher in DR than in ER.

Hollinger et al.[17] showed the unadjusted odds ratio (OR) for death increased considerably with DR (group 2 [4–7 days] = 1.73 [95% CI = 1.00–2.98]; group >8 = 3.42 [95% CI = 1.97–5.96]). Group 2 had an OR = 1.73 and Group 3 had an OR = 3.42. Both of these ratios were significantly higher than 1. However, when the severity of the disease was taken into account, a delay in repair did not predict an increased risk of mortality (group 2 = 1.2 [95% CI = 0.7–2.2]; group 3 = 1.4 [95% CI, 0.8–2.6]), nor did it foreshadow a higher requirement for post-operative extracorporeal membrane oxygen therapy(ECMO) (group 2 = 1.1 [95% CI = 0.5–2.4]; group 3 = 0.5 [95% CI = 0.2–1.4]).

DISCUSSION

CDH is a physiological emergency, not a surgical one. Maintaining cardiorespiratory stability is very important. Irreversible pulmonary hypoplasia and potentially reversible pulmonary hypertension cause infant CDH-related respiratory problems. The balance between these two factors determines the response to therapy. It is ultimately manifested by increased pulmonary vascular resistance and pulmonary artery pressure, right-to-left shunts in the ducts and foramen and progressive hypoxemia. No current proven therapy to increase lung growth exists, so therapeutic interventions aim to manage pulmonary vascular tone.[7,11]

Reviewing the pertinent surgical factors: the side and position of the lesion, the prediction organ size and the degree of hepatic herniation (if present, is essential). The CHD will touch the chest and abdominal cavity organs. The diaphragm hole is patched or closed using either primary repair. Both open or thoracoscopic approaches can be used during surgery.[18]

Nonetheless, the precise definition or criteria for the optimal timing of the surgery are unknown. Due to the heterogeneity of CDH disease severity, some patients can tolerate an ER, whereas others require a period of stabilisation before the appropriate operation can be performed securely. Given the lack of published studies addressing this issue, it was evident that a randomised controlled trial, multivariate analysis, or stratified analysis would be required to determine the optimal timing of surgery for this condition.[16]

Several conditions must be considered when carrying out a CDH repair, including (1) Echocardiographic evidence of pulmonary pressures below 80% systemic; (2) NICU bedside repair until pulmonary pressures are <50% systemic; (3) Repair of severe cases on ECMO within 72 hours once coagulation status is stable. 24-hour pre-repair aminocaproic acid; (4) Mild or moderate cases requiring ECMO: repair following decannulation; (5) A transversus abdominus muscle flap is best for patching left-sided CDH. Prosthetic patches can treat right-sided CDH; (6) Without ECMO repair, nochest tube will be implanted during surgery; (7) Expect a pleural effusion on the operative side and watch for tension and breathing problems; (8) Some CDH patients will develop a postsurgical chylothorax that requires drainage and octreotide. Albumin must replace significant drainage and (9) Monitor immunoglobulins (IgG) weekly if there is considerable drainage and provide intravenous IgG if <200 mg/dL.[15,19–21]

Surgical emergency is the treatment of CDH, and the prognosis depends on the degree of pulmonary hypoplasia. There is much debate and minimal criteria regarding the optimal timing of surgery. The CDH EURO Consortium recommendation of the state that the following physiological parameters before surgery: normal mean arterial pressure for gestation, preductal oxygen saturation consistently 85–95% on FiO2 <0.5, lactate below 3 mmol/L and urine output more than 1 ml/kg/h.[12,22]

Currently, cardiopulmonary stabilisation is followed by definitive surgical repair to treat CDH. The paradigm transition from emergent to DR occurred in 1987 when Sakai and colleagues demonstrated that respiratory system compliance frequently deteriorates after CDH repair.10,23 The study showed thatCDH repair at 24–47 h (early) after birth has the lowest mortality rate. CDH repair at 48–71 h results in less vasodilator medication use and fewer intraoperative complications. In contrast, CDH repair occurring more than 72 hours after birth is associated with numerous chronic pulmonary diseases and longer hospital stays.[16,17,24]

There was no significant difference in longer-term secondary outcomes, such as survival to discharge, when a protocol was implemented that required an echocardiogram-estimated pulmonary arterial pressure of 80% of systemic pressure before CDH repair. This protocol may reduce the incidence of acute post-operative decompensation, but there was no difference in the longer-term secondary outcomes.There is also evidence of a trend for improved 30-day post-operative survival, which did not reach statistical significance.[15]

The first 24-hour OI was temporally reliable and representative of the pre-operative mean (ICC = 0.70, 95% CI = 0.61–0.77). A pre-operative OI of ≤ 9.4 (AUC = 0.95) predicted survival. An increased ventilator day (1.4, 95% CI = 1.1–1.9) and discharge age (1.5, 95% CI = 1.2–2.0) in surgical delay after an OI ≤ 9.4. An OI ≤ 9.4 was reflected in physiologic stability before repair. OI stabilises by 48 h, and DR beyond OI ≤ 9.4 may be associated with a more prolonged ventilator and hospital course without a survival benefit.[13]

The most common way to decide when to fix a CDH is to wait for an unspecified amount of time until the surgeon thinks the patient is "stable" enough.The vague term usually means the patient is stable on the ventilator, has optimal blood flow with minimal pressure support and has little extrapulmonary right-to-left shunting.Previous studies that looked at the effect

of waiting to fix a CDH didnot find a difference in life rates between those who got the problem fixed right after birth and those who did not.[15,19]

The other CDH prediction factorsare pre-natal imaging by ultrasound and MRI, and post-natal factors include birth weight, Apgar score, arterial blood gas, OI, defect size, other severe congenital anomalies, oxygen saturation, vital signs and need for medication.[3,14] Pre-natal imaging usually measures the lung-to-head ratio (LHR), the observed/expected LHR (O/E-LHR), the observed/expected total fetal lung volume (O/E-TFLV), absolute fetal lung volume (FLV), per cent predicted lung volumes (PPLV) and percentage of stomach and liver herniation to predict survival. The O/E LHR of less than 25% and O/E TELV of less than 35% have lower survival. Liver herniation of less than 25% and stomach herniation of less than 50% have significantly higher survival and are associated with defect size. The LHR correlated with liver herniation and defect size.[1,3,25] The presence of a hernia sac improves survival, but morbidity remains controversial. There is no cytogenic anomaly in genetics testing associated with CDH. Right-side CDH is less common and has a worse prognosis than LCDH.[26] No statistically significant difference was associated with survival in birth weight, gestational age (GA) at diagnosis and birth, sex infant and maternal age at left side isolated CDH.[27]

Large-size defects in CDH have a higher risk of significant pulmonary morbidity and long-term pulmonary outcomes than small-size defects. Pulmonary morbidity, including pulmonary hypertension, extracorporeal life support, mechanical ventilation and neonatal intensive care unit days. Long-term pulmonary care, including asthma, rehospitalisation, obstructive lung disease and comprehensive multidisciplinary CDH clinic.[28] Early onset of respiratory distress in neonates with CDH is a poor prognosis and may indicate the presence of more severe pulmonary and cardiac involvement. Pulmonary hypoplasia is an early presentation of respiratory distress. Late respiratory distress has a better prognosis.[29] Late cord clamping results better than immediate clamping at the initial resuscitation manoeuvre. Late cord clamping has a better effect on Apgar score, mean blood pressure and lactate level.[30]

CONCLUSION

The physiological indexes referred to the 3-stability (fluid balanceelectrolyte, temperature and acidbase) and cardiorespiratory stability (OI ≤ 9.4, echocardiogram-estimated pulmonary artery pressure ≤ 80% systemic blood pressure). In detail, the clinical indicators are urine output > 1 ml/kgBW, Fi O2 < 0.5, 95% oxygen saturation, normal temperature, lactate < 3 mmol/L, pulmonary arterial blood pressure less than systemic pressure (≤ 80%) and OI ≤ 9.4. The first pre-operative 24-hour OI mean was temporally reliable and representative (ICC = 0.70, 95% CI = 0.61–0.77). Within any severity level, there were significant differences in 90-day survival or mortality rate between DR and ER (p = 0.002). As a result, there is no optimal timing for surgery in severe cases of CDH. A delay in repair did not predict an increased risk of death, nor did it suggest an increased need for post-operative ECMO. Research shows that regardless of the illness's severity, the timing of CDH repair does not affect the mortality rate.The limitation of this systematic review was no studies addressed existing stability and prognostic factors. Further research will be carried out using 3-stability, cardiorespiratory stability and prognostic factors.

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CONFLICT OF INTEREST

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AUTHORS' CONTRIBUTIONS

HP, DA, PGH, MJ and G prepared the conception and design. HP, DA and MJ drafted the article. PGH and G carried out a critical revision of the article, which is essential intellectual content. HP, DA, PGH, MJ and G approved the final paper.

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Obstructed hemivagina and ipsilateral renal anomaly syndrome in an association with endometriosis: Role of Magnetic Resonance Imaging in diagnosis

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SUMMARY

Obstructed hemivagina and ipsilateral renal anomaly (OHVIRA) is a rare congenital malformation of the female urogenital tract characterized by a triad of uterine didelphys, obstructed hemivagina, and ipsilateral renal anomaly. It was formerly known as Herlyn Werner Wunderlich Syndrome (HWWS). The syndrome usually presents with cyclic pelvic pain following menarche. Endometriosis is a prevalent complication. Magnetic resonance imaging (MRI) helps in diagnosing OHVIRA syndrome and endometriosis due to its high contrast resolution and objectivity. We reported a 13-year-old girl who was evaluated for cyclic pelvic pain after her menarche at 12 years of age. Magnetic resonance imaging (MRI) revealed two separate uterine cavities, services and vaginae, indicating didelphys. The left uterine cavity is filled with fluid, and the left hemivagina is dilated and filled with hyperintense and hypointense fluid on T1 and T2, respectively, indicating blood products. Left hemivagina dilatation implicated the presence of an obstructing vaginal septum. A single left adnexal cyst lesion with blood products was suggestive of an endometriotic cyst. Additionally, the left kidney was absent. A uterine didelphys with left hemivagina obstruction, hematometra, hematocolpos, and the ipsilateral ovarian endometriotic cyst was diagnosed. A final diagnosis of OHVIRA syndrome or HWWS was made, considering that she had no left kidney.MRI is a suitable diagnostic tool for precise anatomical delineation of the uterus, cervix, and vagina in uterovaginal disorders such as OHVIRA syndrome. MRI can also properly evaluate endometriosis and adhesion.

INTRODUCTION

Obstructed hemivagina and ipsilateral renal anomaly (OHVIRA) syndrome, formerly known as Herlyn Werner Wunderlich Syndrome (HWWS), was first reported in 1922. It is a rare condition of congenital malformation of the female urogenital tract involving müllerian and mesonephric duct anomalies. OHVIRA syndrome is a trifecta of ipsilateral renal abnormalities, obstructed hemivagina, and uterine didelphys symptoms.[1] The main clinical manifestations are cyclic pelvic pain and mass following menarche. Pelvic endometriosis and infection are prevalent complications. Early diagnosis is beneficial because this condition can be treated by vaginal septum excision and delay in diagnosis may worsen the associated endometriosis. Magnetic resonance imaging (MRI) is the modality of choice for diagnosing HWWS because of its high contrast resolution and objectivity.

CASE PRESENTATION

A 13-year-old girl was evaluated at our hospital for cyclic pelvic pain localized at the lower abdomen following her menarche (at 12 years old) as well as dark-brown discharge with a flow rate of approximately five menstrual pads per day. Previous medical history indicated no vaginal discharge, nausea, or vomiting before the current condition. Blood investigation revealed no significant changes in erythrocytes (4.63×10^6), leukocytes ($7 \times 10/\mu L$), hemoglobin (13.5 mg/dl), thrombocytes ($317 \times 10^3/\mu L$), prothrombin time (11.1 s; control: 11.0) and activated partial thromboplastin time (32.7 s; control 31.2). Her micturition is normal, with no substantial difficulties. Additional blood investigation revealed normal kidney function (blood urea nitrogen of 6 mg/dL and creatinine of 0.52 mg/dL) and normal blood electrolyte levels (sodium of 139 mEq/L, potassium of 3.7 mEq/L, and chloride of 105 mEq/L). Her family history indicated hymen imperforate in her younger sister.

Physical examination revealed lower abdominal bulging. Her most recent ultrasound examination revealed uterine enlargement, bicornuate, left ovarian cyst (4.95 × 3.95 cm), and uterine hematometra. MRI revealed two separate uterine cavities, cervices, and vaginas, indicating didelphys (Figure 1). The left uterine cavity was filled with fluid, and the left hemivagina was dilated and filled with hyperintense and hypointense fluid on the T1-weighted image (T1WI) and T2WI, respectively (Figure 2), indicating blood products. Left hemivagina dilatation implicated the presence of an obstructing vaginal septum. A single left adnexal cyst lesion with blood products indicated an endometriotic cyst. Additionally, the left kidney was absent (Figure 3). Following the laparoscopy diagnosis, we discovered a didelphic uterus, left hemi uteri (size 10×7×5 cm), right hemi uteri (size 7×5×4 cm), and multiple endometriotic nodules on the peritoneum and diaphragm wall. The patient underwent surgical intervention of septectomy, which consisted of needle aspiration resulting in approximately 150 ml of dark-red fluid. The septectomy was performed at 10 cm in length with ligation and sent to the Pathology Anatomy unit. After 3 days of care, the patient was diagnosed with uterine didelphys with left hemivagina obstruction, hematometra,

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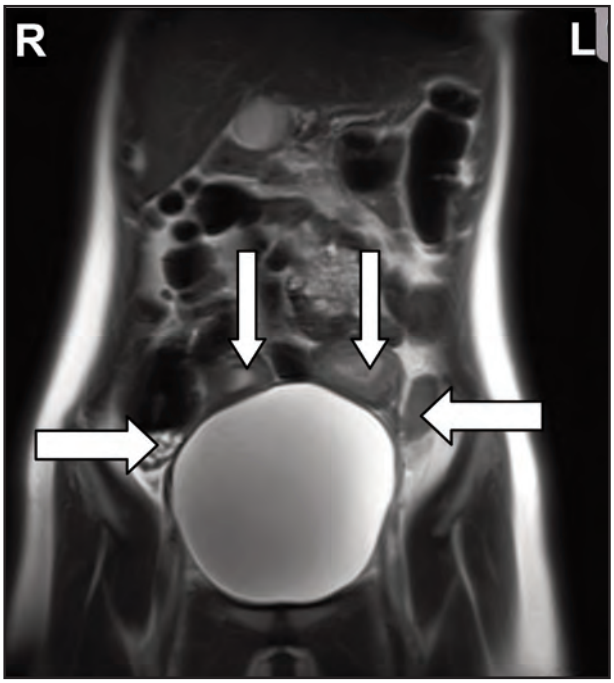

Fig. 1: Coronal T2WI MRI shows two separate uterine cavities (thin arrows) with normal right ovary and left endometrioma (thick arrows)

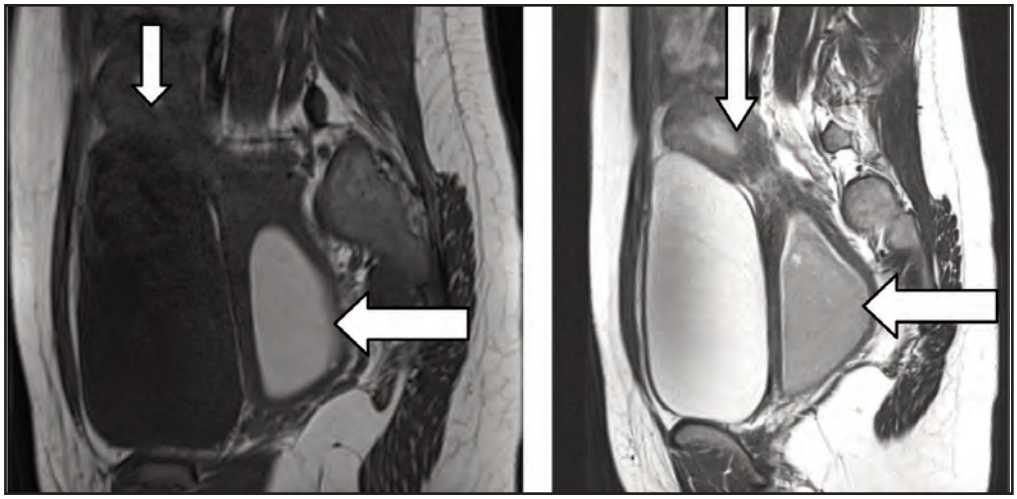

Fig. 2: Sagittal T1WI MRI [A] and T2WI MRI [B] show left uterine cavity (thin white arrows) with dilated hemivagina (thick white arrows) showing a hyperintense signal on T1WI (thick white arrow) and hypointense signal on T2WI/T2 shading (thick white arrow), suggestive of blood products within the uterus (hematometra, thin white arrow), and within the vagina (hematocolpos, thick white arrows)

hematocolpos, and ipsilateral ovarian endometriotic cyst. A final diagnosis of OHVIRA syndrome or HWWS was established, considering that she had no left kidney. No complaints were raised postoperatively, and the patient's daily activities returned to normal.

DISCUSSION

Congenital malformations, also known as Müllerian Duct Anomalies (MDAs), originate from improper müllerian duct development. This may be due to complete agenesis, defective vertical or lateral fusion, or resorption failure. Properly classifying MDA is important because the associated risks of poor pregnancy outcomes and treatment can vary widely among anomalies. The American Society of Reproductive Medicine developed the most prominent classification system.[2]

The OHVIRA syndrome, which was first identified in 1922 and is now known as the HWWS, combines a vaginal septum with type III müllerian anomalies.1 The incidence has been observed in 0.1–3% of various case studies.[3] The classic renal manifestation of OHVIRA syndrome is ipsilateral renal agenesis. Reports also indicated additional kidney anomalies, including crossed fused ectopias, duplicated kidneys, rectovesical bands, and dysplastic kidneys. Renal agenesis is the classic presentation, but other anomalies include renal dysplasia and ectopic ureters.[3,4] The müllerian ducts' lateral

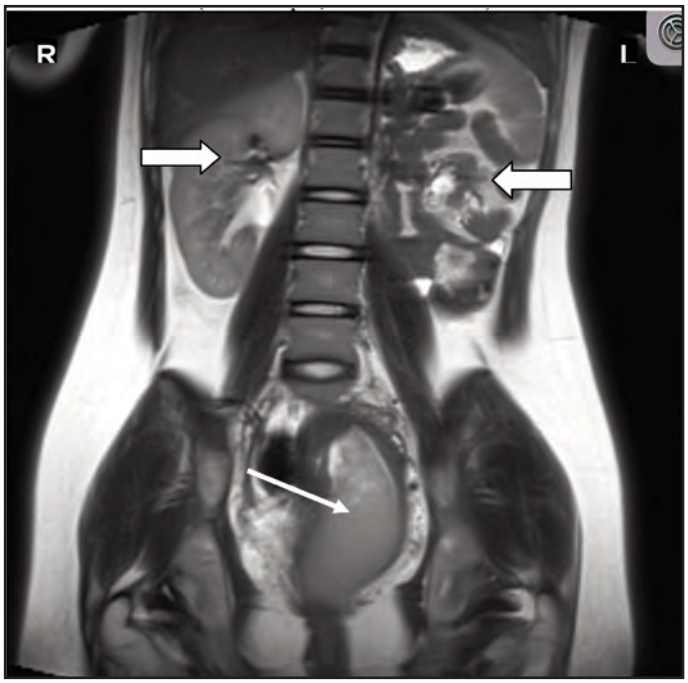

Fig. 3: Coronal T2WI MRI shows dilatation of left hemivagina filled with blood (thin arrows), normal right kidney and absence of left kidney (thick arrows)

nonfusion causes an asymmetric obstruction in uterine didelphys, usually accompanied by renal agenesis on the side of the obstruction.[4] Notably, renal agenesis is located ipsilateral to the dilated uterine cavity in patients with OHVIRA syndrome or HWWS. The caudal parts of the müllerian ducts fuse to form a single double cavity uterus at 8–12 weeks of fetal development. The septum recedes by 20 weeks of pregnancy, resulting in a single cavity. Hence, delayed development of the müllerian and mesonephric ducts at 8 weeks of gestation may cause an anomaly known as uterus didelphys.[5]

Non-specific symptoms, such as recurrent pelvic discomfort or dysmenorrhea caused by increasing distension of the blocked hemivagina, usually accompany menarche. A timely, correct diagnosis is crucial because quick treatment alleviates the problem, prevents additional consequences from chronic obstructed hematocolpos, and preserves reproductive potential. Improper information about the condition, frequent menstruation in the context of incomplete vaginal outlet obstruction, and delayed hematocolpos expansion were all cited for the delays in diagnosis. This case is very encouraging because the patient was diagnosed one year after menstruation. The age of the type of vaginal malformation, obstructed or unobstructed, is important for diagnosis. The average age of diagnosis is 12.8 years in cases with total hemivaginal blockage, compared with 20.6 years in cases of incomplete obstruction.[6]

This patient also had an endometriotic cyst (endometrioma) on one side with hemivaginal obstruction and multiple endometriotic nodules on the peritoneum and diaphragm wall. The relationship between pelvic endometriosis and HWWS remains unclear. Sampson's theory of retrograde menstruation and implantation is thought to explain most issues although no single theory of the etiology of endometriosis can explain all pelvic endometriosis cases. Regarding the association of endometriosis with müllerian anomalies, endometriosis is not more frequent in patients with müllerian anomalies as a whole but is more frequent with müllerian abnormalities with outflow obstruction, hematosalphynx, hematometra, or hematocolpos.[7] Jung et al. revealed that 100% of ovarian endometrial cysts were ipsilateral to the vaginal septum. Long-term continuous reverse menstrual flow has been predictive of pelvic endometriosis onset and progression. HWWS was associated with an increased risk of pelvic lesions.[8]

Ultrasound is frequently used as a screening modality for suspected müllerian duct abnormalities, but it is far inferior to MRI (able to differentiate with T1WI and shading on T2WI) because of its ability to differentiate details (e.g., cysts vs. Endometriosis). MRI rather than computed tomography should follow because the latter involves radiation exposure, and many questions remain unanswered because of its limited soft-tissue resolution. MRI is a good technique for examining the usually complex MDAs. The significant difference in uterovaginal anomalies is associated with multiplanar capabilities, better tissue characterization, and a wide range of views. Precise anatomical delineation of the uterus, tubes, cervix, and vagina is essential. Furthermore, MRI can quickly detect any concurrent renal and urethral abnormalities and define the contents of occluded cavities (e.g., simple fluid vs. blood). The health of these reproductive organs and the presence of endometriosis, pelvic inflammation, and adhesions can be determined through MRI.[9] MRI is an effective diagnostic support tool for identifying anatomical abdominal abnormalities with the right choice of protocol sequences to obtain optimal MRI.[9]

In general, T2-weighted sequences are considered the cornerstone of pelvic MRI because of their superiority in delineating the zonal anatomy of the uterus. T2-weighted sequences should include axial images and those parallel to the long axis of the uterus for better characterization of the external uterine contour, such as the adnexa and ovaries, for MDA classification. Targeted axial or coronal T2-weighted MRI of the abdomen allows assessment of associated urinary tract anomalies. T1-weighted images with excellent soft tissue contrast between the myometrium and the overlying fat may be helpful for characterization. More importantly, fat-saturated T1WI is instrumental in demonstrating blood products of a subacute stage in hematometroclopos or glandular components of deep infiltrative endometriosis.[9,10]

The primary treatment for OHVIRA syndrome is surgical intervention, specifically vaginal septum excision, which removes the blockage. One-stage vaginoplasty, which includes entire septum excision, is performed in a single procedure. Surgery reduces the risk of pelvic endometriosis because of retrograde menstrual seeding.[7] The patient's renal function was normal in this case, but it must always be evaluated because of renal agenesis on the side of the hemivagina obstruction. The history of the younger sister experiencing imperforate hymen needs to be further investigated to determine any recessive inheritance pattern.

CONCLUSION

OHVIRA syndrome or HWWS is characterized by a triad of uterine didelphys, obstructed hemivagina, and ipsilateral renal anomalies. MRI is a suitable diagnostic tool for precise anatomical delineation of the uterus, cervix, and vagina in uterovaginal disorders such as OHVIRA syndrome. Endometriosis, which is a common complication of OHVIRA syndrome, can be properly evaluated using MRI.

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Clinical improvement of diffuse intrinsic pontine glioma treated with radiation therapy concurrent with temozolomide: A case report

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SUMMARY

Diffuse intrinsic pontine glioma (DIPG) is a highly aggressive paediatric brain tumour and nowadays has not had satisfactory result, with most patients do not survive within 1 year of diagnosis. Due to its proximity to critical organs, surgery is avoided, and radiation is the mainstay of treatment. In this case report, we present a case of DIPG treated with radiation and concurrent temozolomide. A 7-year-old child was admitted with complaints of weakness in the eyelid, upper and lower limbs 2 months ago. Physical examination showed tetra paresis and bilateral cranial nerve palsy. Magnetic resonance imaging (MRI) scan showed intracranial tumour consistent with DIPG. Diagnosis was made based on imaging as surgery or biopsy can lead to further morbidity. The patient underwent radiotherapy with concurrent chemotherapy of temozolomide. Radiation was given by dose of 54 Gy/30 fractions (30 × 1.8 Gy) with volumetric arc therapy (VMAT). Due to technical issue after the first five irradiations resulting in 2 weeks delay, boosting of dose by 5 × 1.8 Gy was then given, hence, the total dose was 63 Gy. The booster only targeted the gross tumour volume. Following radiation, the patient felt clinical improvement. Eyelid and limb movement improved since the 15th fraction. At the last fraction, the patient's condition improved symptomatically, but experienced complaints related to post radiation oedema including dizziness and nausea. These complaints were improved upon steroids administration. The MRI evaluation will be done after 8 to 12 weeks of radiation, considering the effects of acute radiation could still occur at this period. In conclusion, a combination of radiotherapy and temozolomide could be an option for DIPG management, with tolerable acute toxicity and possible clinical improvements.

INTRODUCTION

Diffuse intrinsic pontine glioma (DIPG) is a highly aggressive and fatal brain cancer that primarily affects children.[1] It arises in the brainstem, specifically the pons, and has a near 100% fatality rate. A previous study in the United States reported that the incidence of DIPG is estimated to be around 200 to 300 cases per year.[1] Furthermore, DIPG only constitutes approximately 10% of all paediatric brain tumour cases. The DIPGs are characterised by their unique genetic makeup, with nearly 80% of cases harbouring a K27M mutation in either the H3.3 or H3.[1] histone genes. This mutation is found in 78% of DIPGs and 14% of non-brainstem paediatric glioblastomas.[2]

The clinical features of DIPG can vary depending on the tumour location, nature, and growth pattern.3 DIPG can result in the dysfunction of multiple cranial nerves, leading to symptoms such as difficulty swallowing, changes in speech and facial weakness.[3]

DIPG often affects the cranial nerves, leading to various symptoms such as diplopia (double vision) due to abducens palsy (cranial nerve VI dysfunction). Facial weakness or asymmetry may also occur due to damage to cranial nerve VII.[4] DIPG can also cause motor deficits, including ataxia (lack of coordination), dysmetria (inaccurate movements) and dysarthria (difficulty speaking).[3]

The treatment of DIPG remains a significant challenge due to the aggressive nature of the tumour and its location in the brainstem. Focal radiotherapy continues to be the standard treatment for children diagnosed with DIPG, extending overall survival by approximately 3 months. Without radiotherapy, the median overall survival was 5 months.[4] Given the difficulty in treating DIPG, there are ongoing research efforts and clinical trials aimed at understanding the biology of the tumour and developing innovative treatment approaches. The use of concurrent radiation with temozolomide as a form of combination treatment has also been used previously.[5]

We report a case of clinical improvement in DIPG in a 7-year-old child treated with a combination of radiation and concurrent temozolomide.

CASE PRESENTATION

A 7-year-old child presented to the radiation oncology clinic with a complaint of difficulty opening the eyelids for the past 2 months. The complaint was accompanied by difficulty swallowing, occasional choking, as well as weakness and stiffness in the upper and lower extremities. The patient had no significant prior medical history.

Physical examination revealed the child appeared weak, with a condition of ptosis in the eyes, along with an impression of weakness in the upper and lower extremities.

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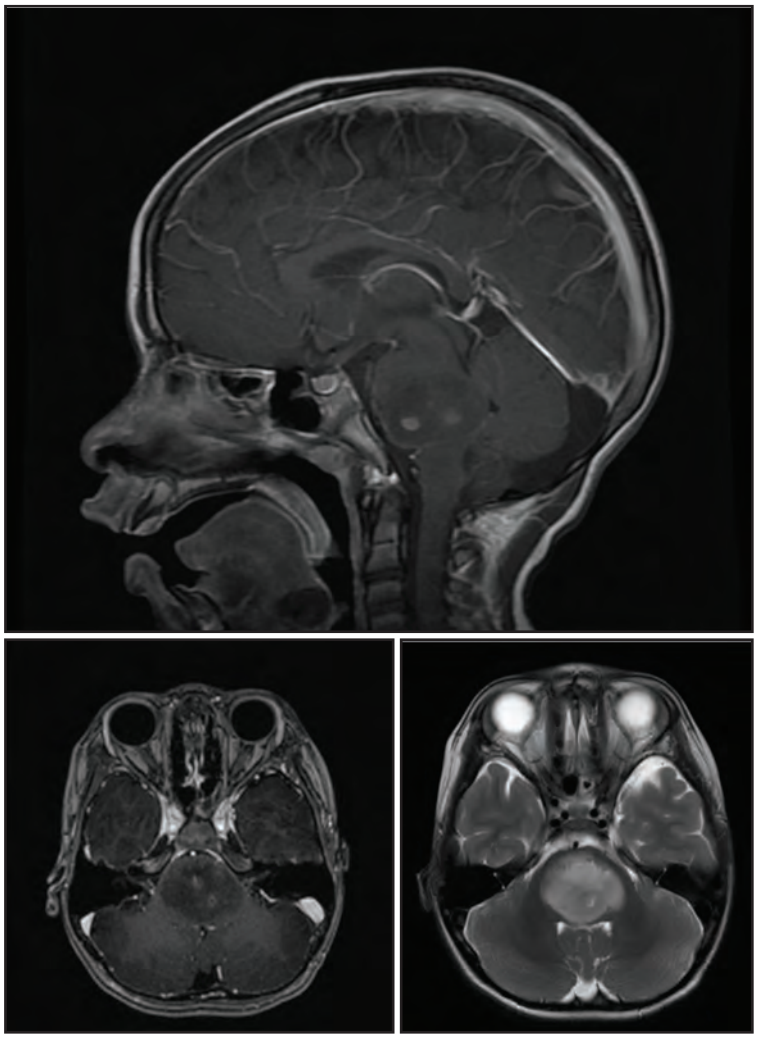

Fig. 1: Appearance of DIPG on T1 and T2 MRI sequences in sagittal and axial sections

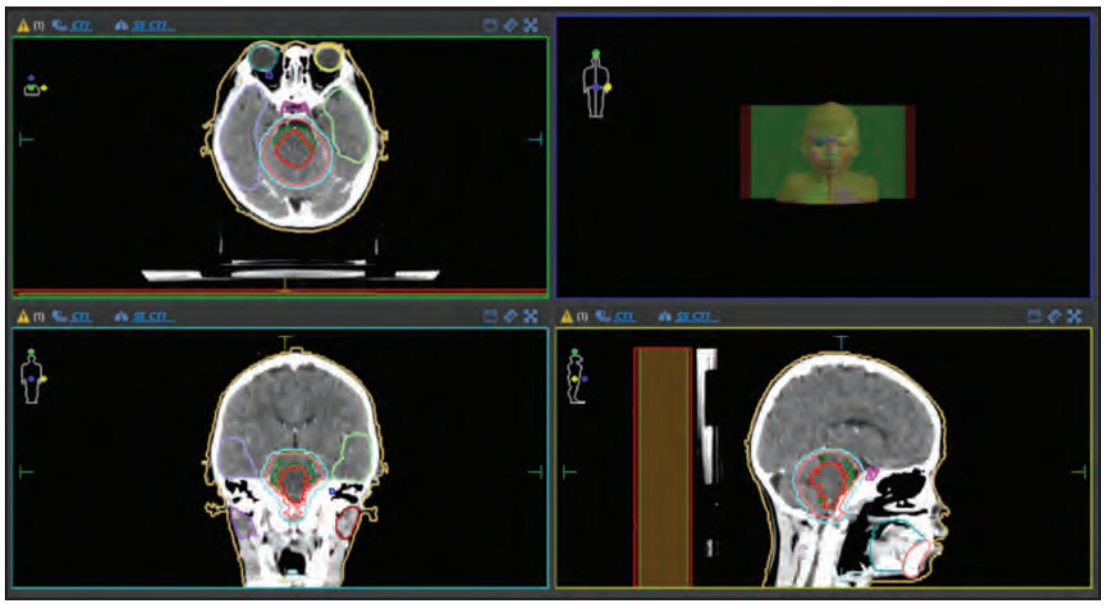

Fig. 2: Contouring in the case of DIPG. The red line indicates GTV, pink shows CTV, and light blue represents PTV

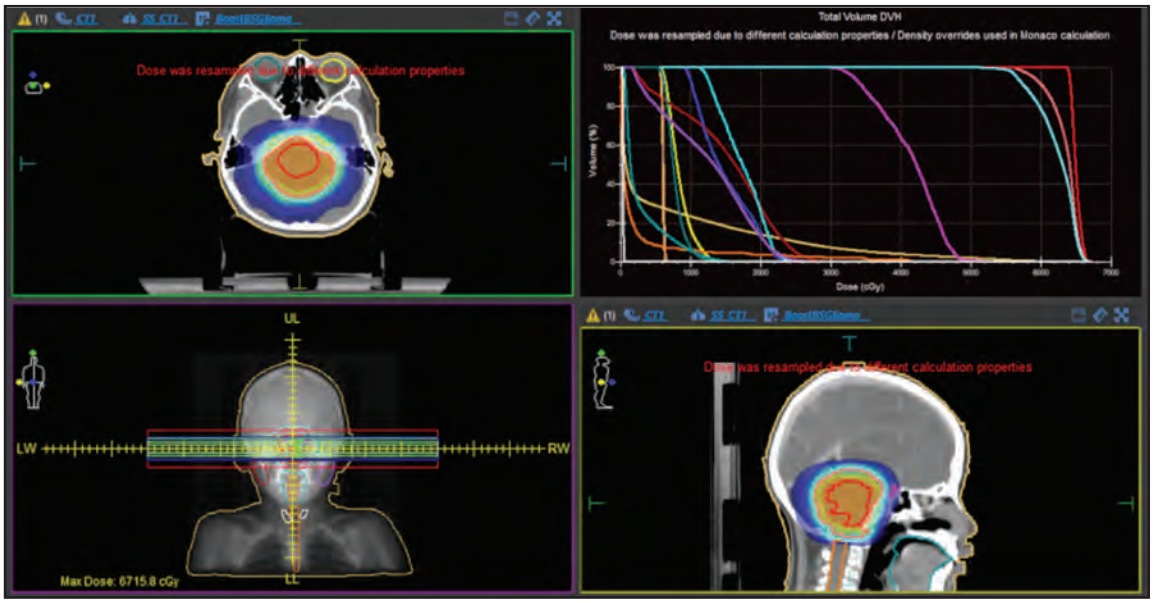

Fig. 3: The result of the TPS dose calculation displayed in the form of a colour wash. The orange or reddish colour indicates the prescription dose/higher dose, while the green or tendency towards blue shows the spread of a lower dose

The patient presented with visual acuity of 6/19 in the right eye (OD) and 6/15 in the left eye (OS) with nystagmus was present. The patient exhibited free ocular movement in both eyes, indicating normal mobility of the eyes without restriction or weakness in extraocular nerves. Examination revealed clear corneas, positive light reflex and clear lenses in both OD and OS. Fundus examination showed clear media in both eyes (OD and OS), papillary diameter measured 0.3-disc diameters in both eyes, and retinal arteriovenous ratio was 2/3 in both eyes. The patient exhibited grade 2 movement in both upper and lower extremities, as per the Medical Research Council (MRC) scale. The child also appeared inactive and difficult to engage in communication but was still seemingly aware of his surroundings.

MRI examination showed an enlargement of the pons due to a mass filling in the brainstem, measuring 3.5 × 3 × 2.3 cm, hypointense on T1 sequence and hyperintense on T2 (Fig. 1). Upon administering gadolinium contrast, there appeared to be several areas that enhanced the contrast heterogeneously. These findings are consistent with a DIPG. No lesions were found in the spinal cord.

The parents have already consulted with a neurosurgeon and a paediatric oncologist and based on the signs and symptoms as well as the MRI findings, a diagnosis of DIPG was established. The neurosurgeon did not recommend performing a biopsy or excision due to the potential morbidity that may be caused. The patient was then referred to a radiation oncologist to undergo radiotherapy. Meanwhile, the paediatric oncologist considered administering temozolomide chemotherapy concurrently with radiotherapy.

Irradiation for this patient was planned with a conventional dose of 54 Gy delivered in 30 fractions (30 × 1.8 Gy). Simulation was conducted with a CT simulator, Canon Aquilion Prime. Image acquisition was performed on the head and neck area. From the CT simulator results, an image was obtained and then exported to the Treatment Planning System (TPS) Monaco Sim version 5.11. Contouring was performed on the target volume, with the outlining of the gross tumour volume (GTV), followed by a radial expansion of 1 cm to form the clinical tumour volume (CTV), and further expansion of 0.5 cm to form the planning target volume (PTV) (Fig. 2). The PTV area will receive radiation of 54 Gy.

Radiation planning was performed using volumetric arc therapy (VMAT) technique with TPS Monaco Plan software version 5.11 (Fig. 3). After the dose calculation by medical physicist with TPS was completed, and constraints were met, the radiation planning data was sent to the LINAC Versa HD machine for the execution of radiation.

Radiation was administered five times a week, every Monday to Friday, so the radiation would be completed in 6 weeks. During the radiation, the patient also received oral temozolomide administered daily throughout the treatment. However, after five sessions, the LINAC machine broke down, requiring extensive repairs, causing a 2-week delay in radiation. After that, the radiation proceeded smoothly without any hindrances until the completion of 30 fractions. At the end of the fractions, considering the radiation gap after the initial five sessions, and to achieve the maximum therapeutic effect, we attempted to escalate the dose by administering a booster for five fractions, so the total dose delivered was 63 Gy/35 fx. The area receiving this radiation booster was only the GTV, without any expansion at all, to minimize radiation effects.

During the radiation, the patient experienced improvement. There was notable enhancement observed in upper and lower extremity strength, as well as improvements noted in swallowing and opening the eyelids. This improvement occurred after receiving about 15 fractions of radiation.

Towards the end of the radiation and up to 2 weeks post-radiation, the patient complained of headaches and nausea, but these conditions improved when administered steroids.

Clinical evaluation is still ongoing, which will be followed by a repeat MRI of the head 8 weeks post-radiation, with the goal of determining the response to radiation.

DISCUSSION

Radiation is currently the primary treatment option for cases of DIPG. The general dose given is 54 to 60 Gy, delivered conventionally. We escalated the dose to 63 Gy to compensate for the radiation delay that occurred at the beginning of the fractions. A previous study has tried to escalate the dose to 70 Gy.[6] This dose escalation certainly has the potential to cause more severe acute effects, but in this case, those effects were relatively controlled with the administration of steroids.

The radiation technique used is the VMAT technique. VMAT is a radiation therapy technique that has been used in the treatment of paediatric brain tumours. It offers advantages such as highly conformal dose distribution and reduced radiation dose to adjacent normal tissue compared to conventional photon radiation therapy.[7] This will reduce excessive radiation to the surrounding normal brain tissue.

Temozolomide is an alkylating agent that has been investigated as a potential treatment for DIPG. A phase II study conducted by the Children's Oncology Group (COG) tested the efficacy of chemoradiotherapy with temozolomide followed by adjuvant temozolomide in children with newly diagnosed DIPG.[5] The study enrolled 63 children, and all patients received temozolomide at a dosage of 90 mg/m²/day for 42 days in combination with radiation therapy with median times to progression and death were 6.1 and 9.6 months. A previous study reported that the median onset of disease progression following irradiation is < 6 months, with a median survival of 10 months.[8] Nevertheless, there was no significant improvement in event-free survival or overall survival compared to single therapy.

Although some trials have not shown significant benefits in terms of survival, in this certain case, temozolomide is the only currently available agent in our region Indonesia for DIPG. Its administration to this patient was relatively well-tolerated, with minimal haematological side effects.

As for the clinical symptom improvement that occurred, it is not yet known whether it is the effect of radiation, temozolomide, or a combination of both. However, this agent is often used in cases of brain tumours in both paediatric and adult patients, as an adjunct to radiation therapy, as it is believed to have synergistic effects and act as a radiosensitiser.[9]

The clinical improvement experienced by the patient, such as increased muscle strength and improvement in cranial nerve palsy, is certainly something to be pleased about. However, it is unknown how long this will continue. As is already known, radiation therapy has not yet been able to provide a curative effect on DIPG. It may be that the improvement experienced is only temporary. As of the writing of this article, the patient is still under clinical observation and will be followed by radiological evaluation with MRI.

CONCLUSION

The combination of radiotherapy with concurrent temozolomide may be an alternative therapy in improving clinical symptoms in DIPG. The long-term effects and their impact on survival still require further follow-up, and more extensive data is also needed.

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Uncommon splenic cysts in paediatric patients: A case series

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SUMMARY

Splenic cysts are uncommon and classified into parasitic and non-parasitic origins. Non-parasitic cysts are further categorised into primary and secondary forms; primary cysts develop congenitally and progress into adulthood and secondary cysts result from factors such as abdominal trauma, infection or ischemia. This case series presents three instances of splenic cysts in children. The first case involves a splenic epidermoid cyst, the second a pseudocyst and the third a splenic epithelial cyst. All patients exhibited an abdominal lump in the left quadrant that increased in size over time, without additional symptoms. The third patient had a history of abdominal blunt trauma a year prior to symptom onset. Treatment approaches varied: the first and third patients underwent total splenectomy, while the second patient underwent aspiration drainage with frozen section analysis and partial splenectomy. All patients, first, second and third, were discharged 6, 3 and 5 days postoperatively, respectively, without complications. Splenic epithelial cyst (SEC) emerged as the predominant primary non-parasitic splenic cyst type, with an unclear pathogenesis. Typically asymptomatic, splenic cysts are commonly detected incidentally during imaging or exploratory laparotomy. Histopathology stands as the gold standard diagnostic method for splenic cysts. Although rare, paediatric splenic cysts should be considered in cases of abdominal trauma. Imaging serves a vital role in diagnosis, guiding decisions between conservative or surgical interventions based on cyst size, symptoms and associated complications.

INTRODUCTION

Splenic cysts are uncommon in children, especially in the youngest age group, while their prevalence is rising as a result of the extensive use of abdominal imaging and nonoperative treatment for splenic injuries.[1] They are traditionally classified as parasitic and non-parasitic origins.[2] Non-parasitic cysts are further categorised into primary and secondary forms, where primary cysts develop congenitally and progress into adulthood, while secondary cysts result from factors such as abdominal trauma, infection or ischaemia.[3] The differential diagnosis is challenging since various spleen-related non-SC diseases, such as inflammatory pseudotumor of the spleen, splenic hamartomas, and splenic abscesses, should be taken into account.[2,3] In this case report, we present three cases of paediatric splenic cysts with different type of cysts requiring splenectomy.

CASE PRESENTATION

Case 1

A 14-year-old male adolescent presented with a lump in the upper left quadrant of his abdomen, accompanied by increasing pain. Despite taking pain medication, the discomfort persisted. Subsequent abdominal ultrasound revealed a uniform hypoechoic mass within the abdominal region. A cystic lesion resembling a splenic epidermoid cyst was identified through an abdominal computed tomography (CT) scan (Figure1). The preliminary diagnosis indicated an intraabdominal mass suspected to be a splenic epidermoid cyst. An exploratory procedure was conducted, uncovering the cystic mass located in the upper-back portion of the spleen. A complete removal of the spleen was performed (Figure 2). Upon histopathological examination, the findings pointed towards an epidermoid cyst in the spleen with cholesterol ester granuloma. The patient's postoperative condition was favourable, and he was discharged after six days.

Case 2

A 14-year-old male adolescent came to the medical facility with a lump in his upper left abdomen, roughly the size of an apple. He didn't have any other complaints over the past 3 years before seeking medical attention. About a year prior to his current complaint, he had experienced blunt trauma to his abdomen. An abdominal ultrasound was performed, revealing a complex cystic mass near the tail of the pancreas that was compressing his left kidney. Subsequent abdominal CT scan indicated a cyst in his spleen that could be an epidermoid cyst, splenic abscess, lymphangioma, or pseudocyst (Figure 1). The provisional diagnosis was an intraabdominal mass suspected to be a splenic epidermoid cyst. The patient underwent a procedure to drain the cyst and a quick analysis during the procedure indicated it was a pseudocyst without signs of malignancy. This was followed by a partial removal of the spleen (Figure 2). The examination of the removed tissue confirmed it was a pseudocyst. The patient was discharged in good condition 3 days after the surgery.

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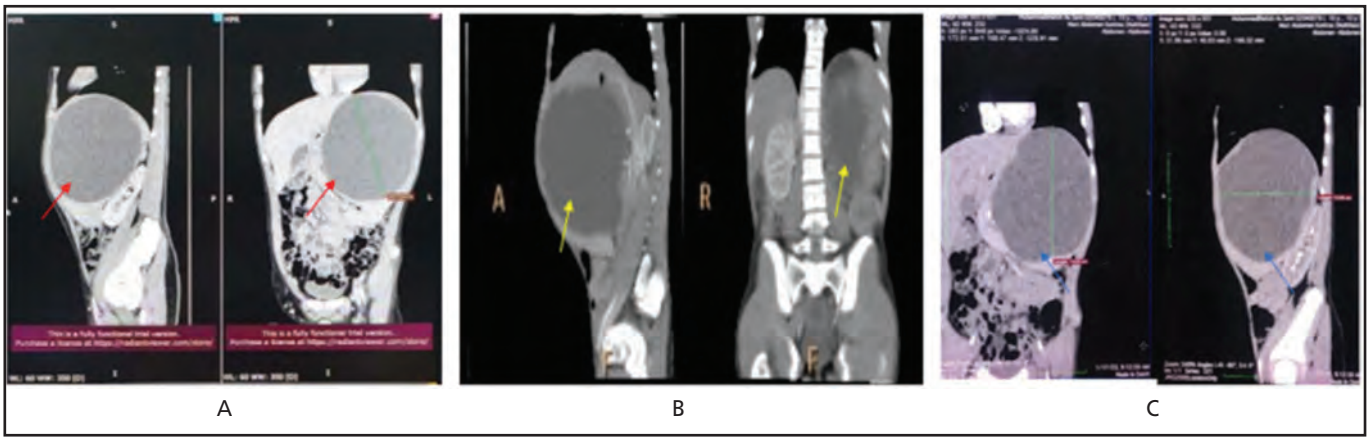

Fig. 1: Abdominal CT scan of case 1, showed splenomegaly with cystic lesion suggested splenic epidermoid cyst with calcification on the wall (A). On abdominal CT scan of case 2, indicated splenic cyst that could be an epidermoid cyst, splenic abscess, lymphangioma or pseudocyst with enlargement to caudomedial (B). On case 3, the cystic mass showed in CT scan exerting pressure on the stomach, duodenum and pancreas (C)

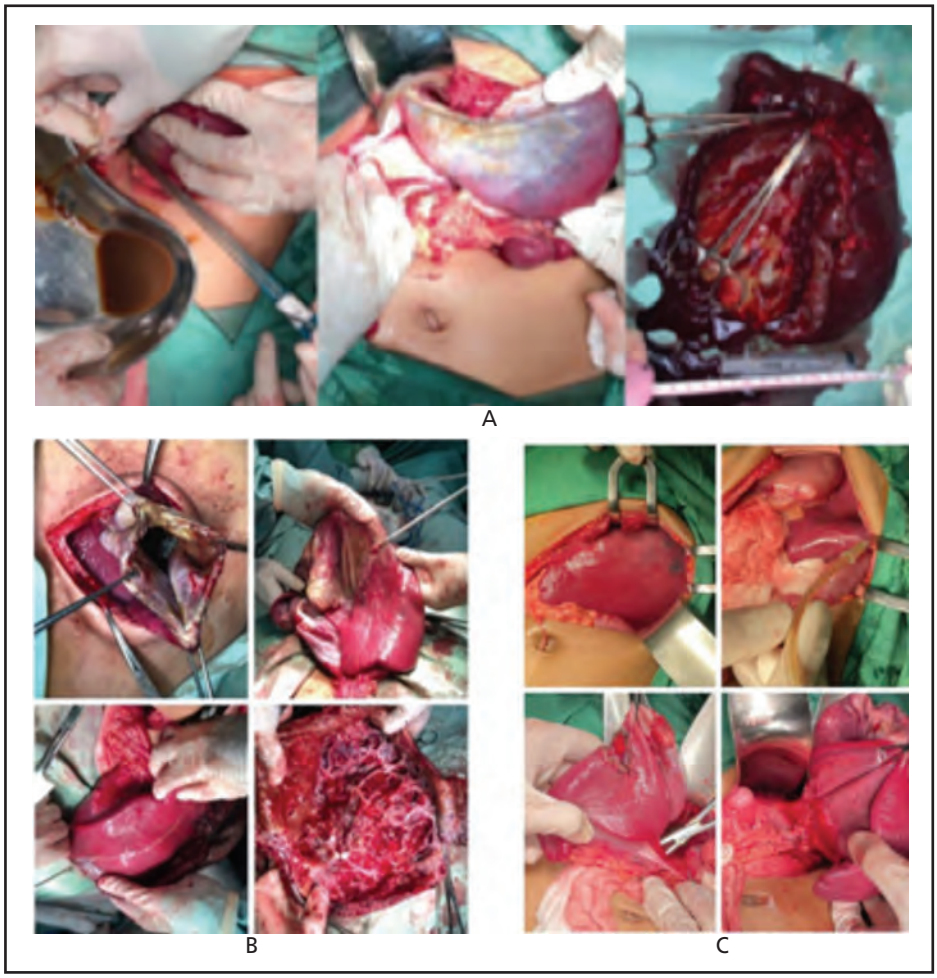

Fig. 2: For case 1, we conduct a laparotomy splenectomy and drainage of the cyst showed brownish fluid around 1300cc (A). The cyst and spleen with separated by clear boundaries in case 2, and we drainage the cyst found serous product around 6000cc (B). In case 3, the cyst wall covers 95% of the spleen with a brownish cystic fluid, so we conducted the total splenectomy (C)

Case 3

A 10-year-old male child arrived with a lump in the upper left quadrant of the abdomen, accompanied by sensations of fullness and loss of appetite. The lump had been progressively growing in size. The patient's medical history included an incident of blunt abdominal trauma a year before the current complaint. An abdominal ultrasound was conducted, revealing a hypoechoic mass situated between the stomach and spleen. Subsequently, an abdominal CT scan displayed a cystic mass within the spleen that was exerting pressure on the stomach, duodenum, and pancreas. This indicated the presence of a splenic epithelial cyst (SEC) (Figure 1). Preliminary diagnosis was an intraabdominal mass suspected to be a splenic epithelial cyst. To address this, a complete removal of the spleen was carried out. The histopathological examination confirmed the presence of a SEC without any signs of malignancy. The patient's recovery was successful, leading to discharge after 5 days following the surgery.

DISCUSSION

We described three cases of paediatric splenic cysts requiring splenectomy. Splenic cysts are rare in children, although their prevalence is increasing due to the use of non-operative abdominal indications of splenic trauma.[1] Splenic cysts are usually seen in the second and third years of life, but they also occur in children and infants. Many factors are discussed about splenic cysts. These congenital diseases go undiagnosed for decades.[1,4] The recurrence rate of this cyst in general is 33% of the total case. Splenic cysts are classified according to Martin's classification, as type I splenic cysts (primary or true cysts), which have a true epithelial lining; and type II splenic cysts (secondary or pseudocysts), which do not have a true epithelial lining and capsule.[5] In the third case, we also examined specific tumour markers to differentiate the cyst from other types such as teratomas, and the results were normal. This finding can distinguish it from other cysts.

Primary splenic cysts are divided into parasitic and non-parasitic cysts. Parasitic cysts are usually seen in endemic areas and are mainly caused by Echinococcus granulosis infestation. Echinococcus granulosis may cause the formation of hydatid cysts, which are sometimes mistaken as epithelial cysts.[4,5] Its can be transmitted through contaminated food, with regions that have high consumption of raw vegetables like salads being at greater risk of infection

Non-parasitic cysts can be divided into congenital cysts and neoplastic cysts. Congenital cysts make up about 10% of all splenic cysts. These cysts can be divided into epidermoid, dermoid and endodermoid cysts. Epidermoid cysts occur due to the inclusion of neighbouring epithelial cells during embryonic development, which then leads to the expansion of the cyst, or from the folding in of the outer layer of cells known as the mesothelium. Dermoid cysts are considered teratoma cysts, containing structures derived from all three germ layers. An endodermoid cyst is a vascular cystic lesion consisting of several ectatic blood vessels. Neoplastic cysts are endothelial origin, include, haemangiomas, lymphangioma, sclerosing angiomatoid nodular transformation (SANT) of the spleen.[3,4]

SEC emerged as the predominant primary non-parasitic splenic cyst type, with unclear pathogenesis. Typically asymptomatic, splenic cysts are commonly detected incidentally during imaging or exploratory laparotomy. Histopathology stands as the gold standard diagnostic method for splenic cysts.[4-6]

Secondary splenic cysts are caused by damage to the spleen or abdominal wall, as well as by splenic infarction or abscess. Distinguishing primary cysts from secondary cysts based on radiographic evidence is challenging due to similarities in features such as calcified or trabeculated cyst walls, peripheral septations, and debris. Splenic cysts caused by injury are not very common, and it is uncertain what the exact frequency of such occurrences might be. It is believed that 75% of secondary splenic cysts, which lead to a splenic hematoma and later develop into an encapsulation with incomplete resorption, are caused by blunt trauma to the upper abdomen. This leads to the accumulation of fluid.[4-6]

Asymptomatic cysts occur in 30 to 60% of patients and are often discovered incidentally, especially those less than 5 cm in diameter. Because cysts grow gradually, they may be observed for years before symptoms appear. Signs and symptoms may include symptoms of pain, swelling, swelling, splenomegaly, or abdominal pressure. The pain may be intermittent or continuous and radiate to the left side, solar plexus, or left shoulder. Splenomegaly causes hypersegregation of the spleen, which can manifest as anaemia, thrombocytopenia and coagulopathy. In our cases, there is no report of any anaemia, thrombocytopenia and coagulopathy before and after surgery.[4,5]

Distinguishing between a true cyst and a pseudocyst using imaging techniques is a challenging task. The cyst can be detected using regular X-ray images if it is significant. A spherical object with a complete and healthy end of the spleen might distinguish between a cyst and an enlarged spleen. Calcification of the arch can also be useful in identifying cysts. On an ultrasound, a splenic cyst typically appears as a clear lesion with smooth edges and shows heightened transmission. If there is flow on Doppler ultrasound, flow lesions such as lymphangiomas and abscesses may be considered. On a CT scan, splenic cysts are observed as uniform, clearly defined lesions and either round or oval in shape. They appear as areas of lower density, not showing significant enhancement or improvement in contrast. The layer surrounding the cyst may become thicker or contain some areas of calcification, and it might sometimes be divided by thin walls. Splenic cysts appear dark on T1-weighted agnetic resonance imaging (MRI) images and bright and consistent on T2-weighted MRI images.[5]

The management of splenic cysts is still controversial. There are no guidelines regarding asymptomatic splenic cysts. Approaches to treat splenic cysts include aspiration and consolidation, internal and external marsupialisation, partial cystectomy (capsule removal), splenectomy and nonsurgical treatment. Symptomatic or complex cysts require

surgical removal.[1-5] Cysts that involve only a portion of the spleen and protruding to the surface can easily be treated by marsupialization, deroofing, or cyst excision. Deep cysts in lower pole that cannot be accessed by this procedure are best treated by partial splenectomy. If the cysts located in the hilar level often requiring complete splenectomy due to bleeding risks. Cyst aspiration as a definitive treatment has been described previously. Agents such as tetracycline or alcohol are injected into the cyst to destroy the cyst lining, but recurrence still occur. Therefore, this procedure is intended only as a temporary management.[7]

We performed splenectomy on three individuals who had splenic cysts. The surgical removal of splenic cysts is typically recommended, due to the risk of infection, rupture from injury, severe bleeding, or the development of abscesses. In the past, the usual approach to deal with splenic cysts involved removing the entire spleen. However, there has been a suggestion to use more cautious methods in order to preserve a greater amount of splenic tissue. In certain cases, total splenectomy may still be recommended, particularly when dealing with polycystic or very large cysts that are mostly concealed within the spleen tissue. This approach is taken to avoid the potential risk of bleeding.[9] Partial splenectomy approach is used to reduce splenic volume (85 to 95%), leaving about 10 to 25% of the normal spleen remaining. The main advantage of partial splenectomy is that it preserves the immune function of the spleen, therefore reduce the risk of post-splenectomy sepsis. The use of laparotomy or laparoscopy can be performed, and generally depends on the skill and preference of the surgeon.[8]

Recovery after total splenectomy is rapid, and most children are discharged from the hospital the day after laparoscopic surgery. Partial splenectomy usually requires the patient to stay in the hospital for 2 to 3 days so that he can be monitored for signs of bleeding. Open surgery requires a slightly longer recovery time to reduce pain, but most children are discharged from the hospital relatively quickly after surgery. To prevent overwhelming post-splenectomy infection (OPSI), antibiotic prophylaxis and immunisation are recommended in the early postoperative period. Vaccinations against Streptococcus pneumoniae, Haemophilus influenzae, and Meningococcus are recommended due to their common association with OPSI. Penicillin is the preferred antibiotic according to most experts, though trimethoprim-sulfamethoxazole can be used as an alternative for those allergic to penicillin. Although there are no definitive guidelines on the duration of prophylaxis, it is generally recommended for at least 2 years following splenectomy. Portal vein thrombosis, occurring in approximately 4.79% of cases, is a relatively rare complication following a splenectomy. Indicators for this condition may include spleen size and thrombocyte counts. Pulmonary hypertension (PH) is another reported complication, with an incidence of about 10%. Although PH is thought to result from thromboembolic involvement of the pulmonary microvasculature through increased thrombus formation, the precise mechanism remains unclear.[10]

CONCLUSION

This study presented three cases of paediatric splenic cysts requiring splenectomy. Despite their rarity in young children, their occurrence is on the rise due to advanced imaging and evolving trauma management. The classification, clinical features, diagnostic challenges, and varied management approaches were explored. From asymptomatic to symptomatic cases, the complexity of paediatric splenic cysts and the nuanced decisions involved in their treatment were highlighted.

ACKNOWLEDGMENT

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CONSENT STATEMENT

All patients involved in this study provided informed written consent. Their identities have been kept confidential throughout the study.

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Radiotherapy for recurrent juvenile nasopharyngeal angiofibroma

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SUMMARY

Juvenile nasopharyngeal angiofibroma (JNA) is a rare paediatric tumour known for its local destructiveness and high recurrence rate. Surgery is the primary treatment modality for JNA, though other options, such as hormonal therapy, embolisation and radiotherapy, exist for inoperable cases. The location of the tumour makes surgical intervention challenging. A 14-year-old male presented with epistaxis and headaches as the chief complaints and was diagnosed with nasopharynx angiofibroma by computed tomography (CT) scan in 2018. Pre-operative embolisation was performed and followed by surgical removal of a 4 cm tumour in January 2019. Pathological examination revealed CD34 positivity, S100 negativity and Ki-67 positivity (5 to 10%), confirming angiofibroma. In October 2019, a 3.6 cm recurrent tumour was treated with embolisation and a second surgery. Pathological findings again confirmed JNA. The patient underwent four surgeries in total, but epistaxis persisted. In 2021, local radiotherapy was administered using intensity-modulated radiation therapy (IMRT) at a dose of 60 Gy in 25 fractions. Serial magnetic resonance imaging (MRI) post-radiotherapy showed a decreasing tumour size, with no further epistaxis and no observed radiation side effects 2 years post-treatment. Radiation therapy remains a strong alternative for managing recurrent JNA.

INTRODUCTION

Juvenile nasopharyngeal angiofibroma (JNA) is a rare tumour of the nasopharynx, around less than 1% of all head and neck cancers, and typically affects young males between the ages of 14 to 25 years.[1,2] It originates from vascular endothelial cells or fibroblasts and has a vasoproliferative nature, resulting vascular and stromal components appearance in pathological findings.[3,4] The primary clinical manifestation of JNA is unilateral nasal obstruction accompanied by epistaxis.

Definitive diagnosis is achieved through angiography, which can also serve as an embolisation therapy.[2] Surgical treatment aims to remove the tumour, but JNA often presents with tumours at the base of the skull, where resection is difficult. This can also interfere with natural facial growth in adolescent patients and lead to higher morbidity rates. Endoscopic JNA could be an alternative to avoid craniofacial alterations. On the other hand, radiotherapy remains an effective primary treatment and adjuvant post-surgery option with residual cases.[5] Therefore, despite being histologically benign, JNA is locally invasive and associated with a high rate of persistence and recurrence, primarily due to incomplete surgical resection. The aim of this report is to highlight the use of a slightly hypofractionated radiation dose of 60 Gy in 25 fractions in a highly recurrent case of JNA.

CASE PRESENTATION

A 14-year-old male came with epistaxis and headaches as a chief complaint, the first computed tomography (CT) scan in 2018 showed nasopharynx angiofibroma, Andrews staging type 2 with destruction at the nasal cavity and left ethmoid (Figure 1).

Preoperative embolisation was performed, followed by surgery to remove a 4 cm tumour in January 2019. The margins were not entirely clear, with some evidence of residual tumour. Pathological findings with CD34 positive, CD31 positive, S100 negative and Ki-67 5 to 10% positive suggested angiofibroma.

In October 2019, 3.6 cm tumour recurred with epistaxis, and another embolisation followed by surgery was performed for a second time (Figure 2).

Pathological findings also suggested juvenile angiofibroma. Residual disease persisted, and a total of four surgeries were performed. Despite these efforts, epistaxis continued to occur, necessitating several blood transfusions. Local radiotherapy was performed using IMRT dose 60 Gy in 25 fractions in 2021.

The target for radiotherapy was the local tumour fuse with Magnetic resonance imaging (MRI) as gross tumour volume (GTV) with a 5 mm margin as planning target volume (PTV) and was performed using IMRT (Figure 4).

A total dose of 60 Gy is divided into 25 fractions. All organs-at-risk (OARs) doses were within the tolerance limits of conventional dose constraints, with maximum doses of adjacent organs as follows: right eye, 2116.4 cGy; left eye, 2729.3 cGy; and pituitary gland, 4921.4 cGy. The mean haemoglobin level during this treatment is 13.5 mg/dL. Serial

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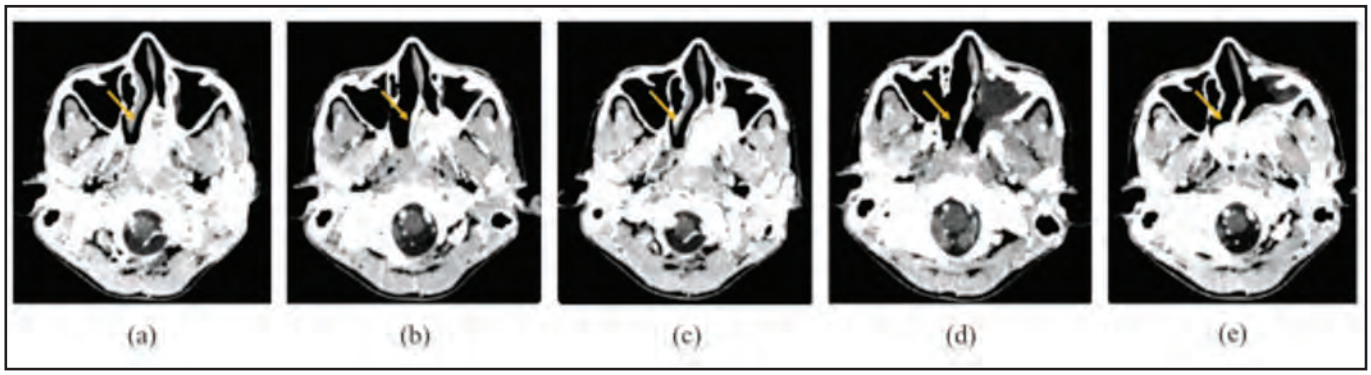

Fig. 1: Serial multislice computed tomography (MSCT) before radiotherapy: (a) pre-operative 1 in 2018, (b) pre-embolisation pre-operative 2 in 2019, (c) post-operative 2 in 2019, (d) post-operative 3 in 2020, (e) post-operative 4 in 2021. Orange arrows indicate the tumour

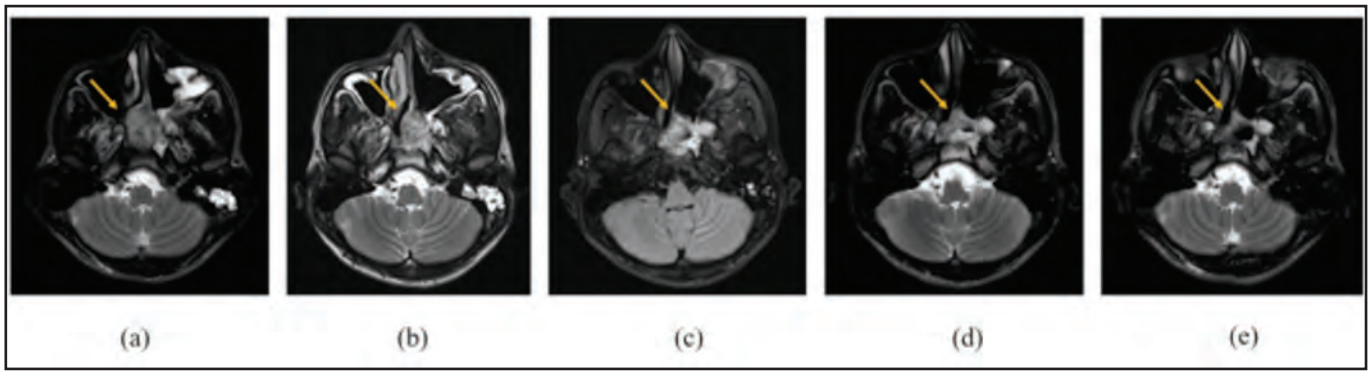

Fig. 2: Serial magnetic resonance imaging (MRI) T2 images showing tumour size: (a) Pre-radiotherapy (RT) (AP 3.7 × LL 2.6 × CC 2.73 cm), (b) 6 weeks after RT, (c) 6 months after RT, (d) 12 months after RT, and (e) 18 months after RT (AP 2.5 × LL 2.13 × CC 1.3 cm)

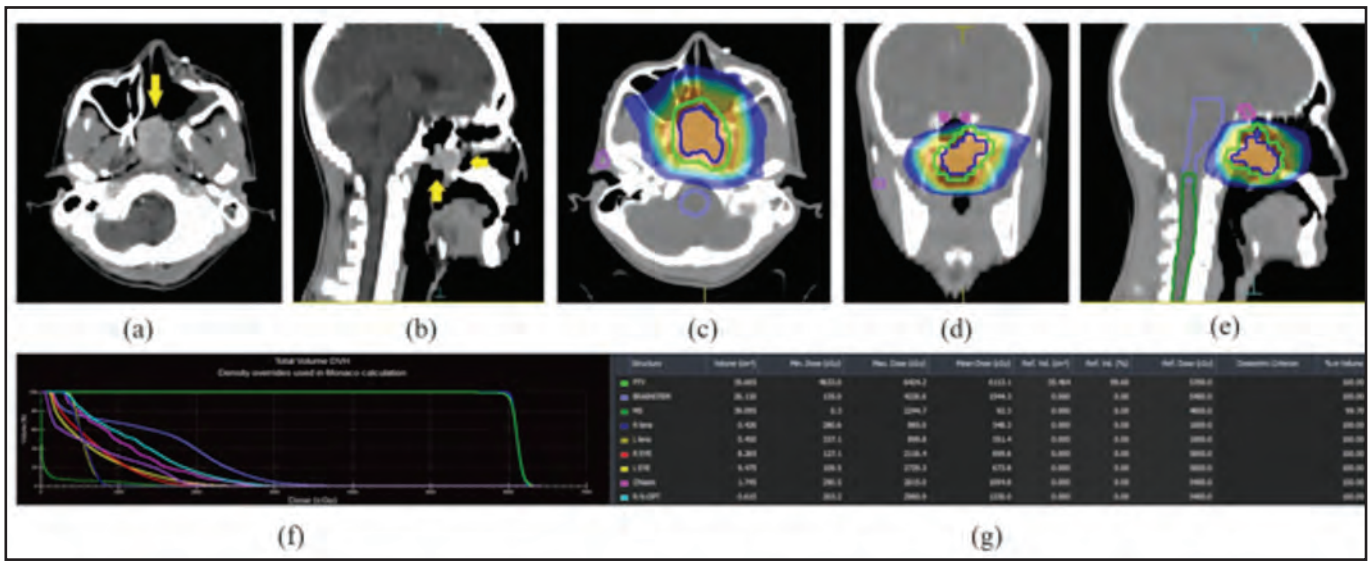

Fig. 4: Illustration of computed tomography (CT) simulation showing the location of the tumour in (a) axial and (b) sagittal views; delineation and dose distribution in (c) axial, (d) coronal and (e) sagittal views. Gross tumour volume (GTV) marked in blue, planning target volume (PTV) marked in green (expanded 5 mm from GTV); (f) dose volume histogram, and (g) dose volume statistic

MRIs were performed and always showed a decreasing size tumour even after 18 months post radiotherapy with no more epistaxis happening and no radiation side effects observed. Additionally, no growth retardation, panhypopituitarism, temporal lobe necrosis, cataracts and radiation keratopathy were observed after follow-up.

DISCUSSION

Treatment options for JNA are surgery and embolisation.[4,5] In this case, the patient already went for four surgeries with prior embolisation, each of them confirmed pathologically as JNA, followed by the recurrent chief complaint which was epistaxis. The surgical approach is still the first-line treatment for JNA, which is benign, proliferative and destructive.[5]

Our following approach for this case was radiotherapy given locally with the IMRT technique preferred over stereotactic radiosurgery (SRS). There are two kinds of approaches for radiotherapy conventional dose or SRS (hypofraction). Stereotactic radiosurgery is still controversial due to the tumour margin being usually unclear.4 In this case, a slightly hypofractionated radiation dose of 60 Gy in 25 fractions was selected due to the patient's highly recurrent JNA and persistent rebleeding despite prior surgeries and embolisation. This aggressive recurrence pattern necessitated a more intensive treatment approach to achieve long-term control and prevent further relapses. A previous study has shown that the radiation dose used was variable, ranging between 3,000 and 5,500 cGy, with a 2-year local control rate of 87.5% and no remarkable adverse effects.[5] Additionally, the tumour's size and anatomical location in our case posed significant challenges for complete surgical resection, requiring a higher radiotherapy dose to ensure adequate tumour control and minimise the likelihood of further recurrence. Radiotherapy is the last treatment option for JNA due to its possibility to induce secondary malignancy.[6] A previous study using proton radiotherapy reported considerable tumour shrinkage and complete remission in five out of ten patients with JNA, with the potential to provide partial or complete symptom relief.[7] However, our facility was not able to administer proton radiotherapy treatment. Additionally, proton therapy is often limited to only few medical facilities in other countries and may not be accessible to all patients. Nevertheless, in our case, the dose was also used to effectively control bleeding. Follow-up examinations and serial MRI over 18 months showed no late complications. The tumour size has decreased significantly with more necrotic regions and no further episodes of epistaxis. The MRI was performed 6 weeks after radiation, every 6 months after radiation for 2 years, and will still be performed annually or if the patient has symptoms in the future.

Based on this patient, there are a few things that can be evaluated from this case. Treatment of JNA should be done directly due to uncontrolled epistaxis could lead to hypovolemic shock. Multidisciplinary team management is important for planning and evaluating how and when to use which modalities are available in our centre. The MRI is the best non-invasive modality to evaluate disease and treatment side effects of JNA. Even though radiation-induced malignancy is still a mystery the incidence varies only from 3 to 26% in >20 years.6 Radiation dose and technique for JNA should be explored more with randomized clinical trials.

CONCLUSION

Radiotherapy can still be a reliable alternative for JNA despite its considerably small potential to induce secondary malignancy. The successful use of a slightly hypofractionated radiation dose in a highly recurrent case of JNA underscores the potential benefits and safety of higher doses in specific clinical scenarios. Radiotherapy doses and techniques for JNA are selected based on clinical judgment considering clinical symptoms, location, size of the tumour, and radiotherapy equipment owned by the centre.

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CASE REPORT

An uncommon case of retinitis pigmentosa patients based on clinical and genetic study

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SUMMARY

Inherited retinal dystrophy (IRD) is a group of phenotypes caused by mutations in visual pathways-related genes, mostly occurring at photoreceptors. This heterogeneous group includes retinitis pigmentosa (RP) recognised by bone spicule at the peripheral retina and the other is Stargardt with macular pisiform flecks. In this study, a 20-year-old male patient with RP symptoms was accompanied by a yellowish pisiform flex in the macula. However, his brother, mother and aunty have typical Stargardt disease. This study involved four persons, two males (cases 1 and 2), their mother (case 3) and aunt (case 4). Initially, cases 1 and 2 came to the clinic, case 1 was diagnosed as RP and macular dystrophy, and case 2 was diagnosed as Stargardt disease. On the follow-up, cases 1 and 2 as well as their father, mother and other family members underwent comprehensive eye examination, including fundus, Snellen, OCT, OCT-A and HFA, and found an uncommon macular abnormality besides typical RP appearance in case 1. The father is healthy while the mother and one of his aunties were diagnosed as Stargardt. A genetics analysis was conducted in case 1, finding various mutations associated with IRD mutation at the cone protein-encoded gene that concentrated at the central and rod protein-encoded gene concentrated at the peripheral retina. Whether the combination of multiple or the same mutations is responsible for this RP phenotype needs further analysis and validation. Cases 2 and 3 genetic analysis showed similar mutation results but with a healthy peripheral retina and only represented Stargardt. Case 1 is considered as RP with macular dystrophy, while cases 2, 3 and 4 are confirmed as Stargardt.

INTRODUCTION

Inherited retinal dystrophy (IRD) is a group of diseases with heterogeneous manifestations and genetic backgrounds. So far, 281 genes have been associated with IRD (https://web.sph.uth.edu/RetNet/home.htm). The damaged retinal cells compromise the patient's visual field partially or completely. At least 20 IRD sub-types were identified, including retinitis pigmentosa (RP), Stargardt, rod-cone dystrophy (RCD), Leber Congenital Amaurosis (LCA), etc.[1]

The clinical features of IRD can be varied among individuals. The key features of each type of IRD are unique. RP is recognised as a condition characterised by arteriolar attenuation, retinal pigmentary changes (hypopigmentation/hyperpigmentation of bone-spicule and pigment clumping) and waxy disc pallor caused mostly by mutation of *USH2A* or *RHO*. Patients with Stargardt disease present pigment mottling, frank macular atrophy, a bull's eye maculopathy and fundus flecks, which are mostly caused by *ABCA4* mutation.[2]

These genetically heterogeneous retinal dystrophies present significant challenges in predicting the causative mutation since mutations can be expected in any of 8 to 61 genes. The high resolving power of whole exome sequencing (WES) solves almost all IRD cases.[3] The remaining unsolved cases are suggested to undergo further genetic analysis, such as whole genome sequencing (WGS) or multiplex ligation-dependent probe amplification (MLPA). The *ABCA4* mutation is well established causing the Stargardt phenotype[4] and limited RP.[5] *ABCA4* mutation is found in around 1 to 250,000 RP cases and causes an RP subtype 19 (RP19; MIM: 601718).6

CASE PRESENTATION

This study involved a family consisting of four persons, two male sons (cases 1 and 2), their mother (case 3) and aunt (case 4). Initially, a 20-year-old male (case 1) visited the eye clinic at the Sardjto General Hospital for evaluation, with a referring diagnosis of hereditary eye disease based on findings seen during a fundus examination. Case 1 suffered from tunnel vision and blurry night vision began 13 years ago. Case 2, a 27-year-old male, the brother of case 1 had blurry vision which was not corrected with an eyeglass and inconveniences under bright light starting from 14 years ago. Case 2 also underwent a fundus examination after finding that case 1 was RP. In further examination of those patients and their family members found that case 1 was confirmed as RP and case 2, his mother (case 3) and aunt (case 4) were confirmed as Stargardt. These patients have a family history of inherited retinal dystrophy shown in their pedigree, which implies that the traits are carried from the maternal lineage. At the first eye evaluation, case 1 visual acuity was best

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Table I: The summary of the clinical assessment of IRD patients' family in this research

Parameters	Case 1, son	Case 2, son	Case 3, mother	Case 4, aunt	father	Aunt
Diagnosis	RP-macular dystrophy	Stargardt	Stargardt	Stargardt	Healthy	Healthy
Sex	Male	Male	Female	Female	Male	Female
Symptoms	Blurry vision, inconvenience at bright light	Blurry vision, inconvenience at bright light	Blurry vision	Blurry vision	Healthy	Healthy
Quality of life	Disturbed	Amenable	Amenable	Amenable	normal	normal
ETDRS	1/60 1/60	6/60 6/60	1/60 1/60	1/60 1/60	normal	6/6 6/6
Macula	Pisiform fleck	Pisiform fleck	Pisiform fleck	Pisiform fleck	Healthy	Healthy
Peripheral	Bone spicule	Clear	Clear	Clear	Clear	Clear
Possible causative mutations	SLC7A14, RHO, ABCA4	ABCA4	ABCA4	-	-	

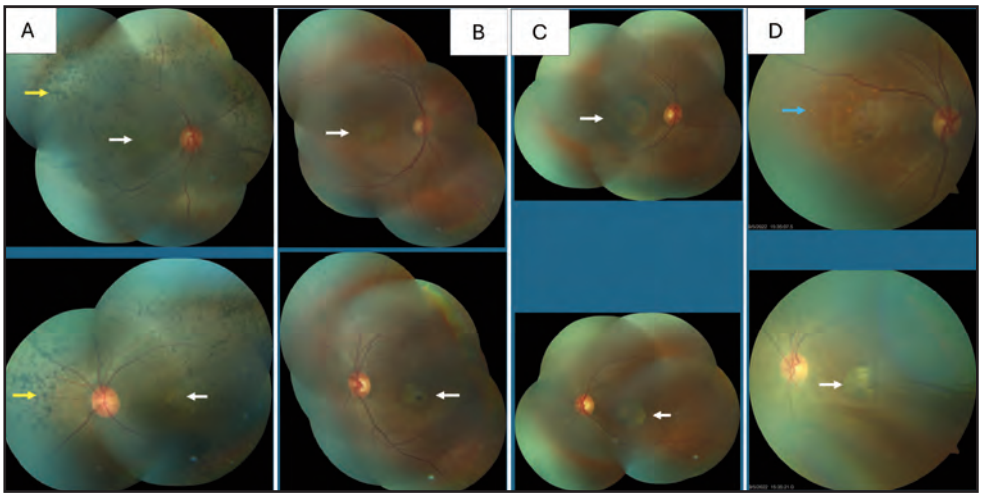

Fig. 1: The fundus image of the studied family. (A) Fundus with pisiform flecks across the macula (white arrow), in both eyes, the patient had a bone spicule in the periphery retina (yellow arrow). (B to C) Stargardt patient, brother (case 2), mother (case 3) has pisiform flecks in both eyes, and (D) aunty (case 4) has lipofuscin accumulation in the macula right eye (blue arrow) and pisiform fleck in the left eye (white arrow) with healthy peripheral retina.

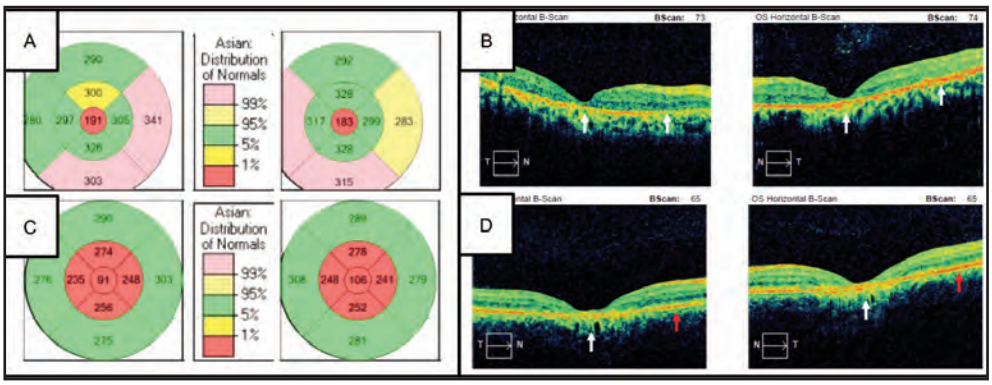

Fig. 2: The OCT image shows the abnormality of the macula as well as the periphery retina of the retinitis pigmentosa-macular dystrophy in case 1. (A) The central red circle indicates a central macular thickness (CMT) which is thiner than normal and (B) white arrow indicates a disturbed photoreceptor layer across the retina. The OCT image shows the abnormality of the macula of Stargardt in case 2. (C) The central and surrounding red circles indicated a central macular thickness (CMT) which thinner than normal and (D) The white arrow indicated disturbed while the red arrow indicated normal photoreceptor layer.

corrected to Snellen 1/60 in both eyes. Anterior segment examination was unremarkable. Fundus and OCT tests initially suggest that case 1 was RP with macular dystrophy. At the follow-up, all subjects underwent comprehensive ophthalmic tests at Sardjito General Hospital. During examination, blood samples and baseline data were also collected. The ophthalmic test was initiated with dilation using topical tropicamide (1%) and phenylephrine hydrochloride (2.5%). The imaging studies were conducted using wide-angle colour fundus image (serial no.: 1103524, VISUCAM NM/FA, Zeiss), optical coherence tomography (Serial No.: 5000-21320, Cirrus 5000, Zeiss) and Humphrey field analyser 3 (HFA) (Serial No.: 860-18955, HFA 3, Zeiss). DNA was isolated from blood samples using QIAamp DNA Mini Kit (Qiagen; Cat.No.: 51306). The library was prepared according to the Illumina protocol. The library was sequenced using Nextseq 550.

Case 1 had a macular abnormality. Dilated fundus examination revealed a pattern of yellow pisiform flecks across the macula, sparing the juxta-papillary region in both eyes. The foveal region exhibited a hyperpigmented appearance and bone spicule in the peripheral region (Figure 1A). Case 2 also showed pisiform fleck sparing the juxta-papillary region in both eyes. The foveal region exhibited a hyperpigmented appearance while the periphery is healthy (Figure 1B).

Case 3 was 53-years-old. She was adapted to her condition and invited to the hospital after her two sons (case 1 and 2) were diagnosed with IRD. She was diagnosed as Stargardt. Her visual acuity was best corrected to Snellen 1/60 right (RE) and 0.5/60 left eye (LE) (Table I). Dilated fundus examination revealed a pattern of yellow pisiform flecks across the macula, sparing the juxta-papillary region in both eyes (Figure 1C). The foveal region exhibited a hyperpigmented appearance. Case 4 was 47-years-old and adapted to her condition; she was invited to the hospital after her two nieces (case 1 and 2) who were diagnosed with IRD. She was diagnosed as Stargardt. Her visual acuity was best corrected to Snellen 1/60 in both eyes (Table I). Dilated fundus examination revealed a pattern of pisiform and yellow flecks across the macula's right eye with a pisiform fleck in the left eye (Figure 1D).

Another aunt of cases 1 and 2 was healthy. No signs of abnormalities were found during her fundus examination. After finishing the eye examination of all family members, they get an education regarding the conditions. The affected member was educated to avoid direct sunlight and received a vitamin. Further follow-up will be conducted to reassess their condition the disease progression.

The second assessment uses The OCT scan for the right (RE) and left eye (LE) of cases 1 and 2. The analysis shows a low CMT score in both eyes (RE: 191 and LE: 183), signifying a decrease in foveal thickness. Inferior and Nasal of RE and LE underwent thickening (Figure 2A). The OCT in case 2 shows an even wider decrease in foveal thickness (CMT RE: 91 and LE: 106) and parafovea (274 and 256) perifovea (235 248) (Figure 2C).

Irregular photoreceptor form was seen in the case 1 (Figure2B). For case 2, irregular in the central while the periphery is normal. Specifically, it shows an irregular photoreceptor form, and the inner foveal pitch is gone at the macula (Figure 2D).

Interestingly, the visual field analysis showed a significant visual field defect, especially in the inferior-temporal region in both the right and left eyes of case 1. The inferior-temporal region undergoes constriction (Figure 3A and B). In case 2, the perimetry analysis unfortunately showed a high rate of fixation losses. However, it showed a defect in almost all peripheral fields, but mostly in the inferior and nasal regions.

The genetic analysis using whole exome sequencing was performed to elucidate the causative mutation of the RP case. After sorting, around 200 variants associated with IRD were found. Then, further filtering was conducted to exclude variants not associated with the RP and Stargardt.

Notably, ABCA4, USH2A, FLVCR1, SLC7A14, RP and RHO were reported to cause IRD cases in the population. RHO was associated with autosomal dominant RP. The found mutation was heterozygote but not in the regulatory region. The FLVCR1 (Feline Leukaemia Virus Subgroup C Receptor 1) gene encodes a protein that exports heme and regulates intracellular heme concentration. This gene was reported with syndromic RP.

The SLC7A14 is responsible for cationic amino acid transporter. Mutations in SLC7A14 were reported to cause autosomal recessive RP in Chinese patients.[7] Knockdown of slc7a14 in zebrafish results in peripheral photoreceptor defects, which are most likely from the rod cell.[8] A Slc7a14 knockout in mice led to a thinning retinal layer and compromised electroretinography (ERG).[7] CRISPR–Cas9–mediated knock in p.Gly330Arg mice not only shows a thinning retinal layer but also auditory impairment.[9] The genetic analysis of case 2 and 3 was then filter out other mutations besides *ABCA4*. The exact causative mutations should be interpreted carefully in the future.

DISCUSSION

After conducting clinical and genetics assessments of three IRD cases, the results showed interesting findings. Fundus examination of case 1 showed three main features of RP including bone spicule, attenuate vascular and pallor optic disc with a yellowish fleck in the macula. OCT scan showed a decrease in foveal thickness in both eyes. Visual field tests also found defects, especially in the inferotemporal aspect. His brother (case 2), mother (case 3) and aunt (case 4) had Stargardt's main feature and pisiform flex. Genetic tests of case 1 showed multiple mutations at both RP (SLC7A14, RHO) and Stargardt (ABCA4) pathological gene. Cases 2 and 3 had many mutations in Stargardt i.e. ABCA4 and EVOL4. The mode of inheritance of IRD needs to be studied further. The mother and aunty do not have an RP feature. The RP in case 1 was most possibly in recessive form. The case 1 most likely resulted from genetic interplay between SLC7A14 and ABCA4, with missense mutations at ABCA4 found together with heterozygote SLC7A14.

The family history plays a part in this case. First, the vision impairment of the mother and aunt was not treated and neglected then they were unaware of sibling vulnerability of having the same diagnosis. This results in a more severe condition in the case 1 and 2. A serious care such as avoiding direct sunlight and vitamin A supplementation might postpone the onset of IRD condition. This care also halts the progression of IRD.

Special attention was given to case 1 because several IRD cases might exhibit similar late-stage phenotypes, with features such as severe retinal cell death, extensive atrophy of the retina, and irreversible visual loss. However, RP and Stargardt2 had two differentiated diagnoses and pathways. Peripheral visual loss and inconvenience under bright light are most likely due to rod cell death in the rear retina since the rod was more abundant in the rear and the function of a dime light acceptor. Noticed RP cases with a shared genetic background with Stargardt should be interpreted cautiously. This case is most likely under recessive inheritance due to non-RP parents. The previous study also reports the different IRD subtypes expressed in the same offspring.[10]

CONCLUSION

In this study, an uncommon case of retinitis pigmentosa (RP) with macular lesions is described which is most likely in a recessive fashion. This family is affected by two subtypes of Inherited retinal dystrophy, RP and Stargardt.

ETHICS STATEMENT

The study was approved by the ethical committee of the Faculty of Medicine, Public Health, and Nurses, Universitas Gadjah Mada (No.: KE/FK/1315/EC/2021). The retinitis pigmentosa patient and family were examined at Sardjito General Hospital, Yogyakarta.

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