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Original Article

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Assessment of Second Trimester Genetic Amniocentesis: A Review of 6 Years of Experience at Sanpatong Hospital, A Mid-level Secondary Hospital Setting

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ABSTRACT

OBJECTIVE This study aims to assess the indications, complications, and outcomes of second-trimester genetic amniocentesis performed at Sanpatong Hospital, Chiang Mai, Thailand.

METHODS A cross-sectional descriptive study analyzed data collected from high-risk pregnant women who underwent second-trimester genetic amniocentesis at Sanpatong Hospital between October 1st, 2016 and September 30th, 2022. The data include indications for the procedure, complications, and pregnancy outcomes.

RESULTS A study of 451 women with high-risk pregnancies who underwent amniocentesis found that the most common indications for second trimester genetic amniocentesis were advanced maternal age (49.4%) and a high-risk Quad test (49.4%). Abnormal chromosomes were detected in 3.1% of cases, with aneuploidy the most common type (2.1%), primarily trisomy 21 (1.3%). The overall aspiration success rate was 100%. The only complications related to the procedure were pelvic pain (0.6%) and placental hematoma (0.2%). There were no fetal losses within 30 days after amniocentesis. The culture failure rate was 1.1%. Pregnancy outcomes included preterm delivery (12.3%) and normal term delivery (87.7%).

CONCLUSIONS Performing second trimester genetic amniocentesis at Sanpatong Hospital, a mid-level secondary hospital, over a six-year period resulted in no fetal losses.

KEYWORDS amniocentesis, second trimester, genetic, chromosome abnormalities

INTRODUCTION

Amniocentesis is a prenatal diagnostic procedure that involves withdrawing amniotic fluid from the amniotic sac for cell culture (1). The resulting cells are then separated and analyzed for chromosomal abnormalities in the fetus. This procedure was first performed in the 1950s and was used to determine fetal sex (2). Amnionic cell culture and testing of fetal karyotypes was first successfully used in 1966 (3). Subsequently, amniocentesis became a standard procedure for diagnosing various chromosomal abnormalities.

The optimal gestational age for performing amniocentesis is between 15 and 20 weeks of pregnancy (4). Previous studies have, however, have reported complications associated with this procedure including abdominal cramping, pelvic pain, membrane leakage, infection, and fetal loss (5-8). Nevertheless, second-trimester genetic amniocentesis is considered safe when realtime

ultrasound guidance is employed for needle placement (8).

Despite inherent risks, amniocentesis has remained a crucial diagnotic tool for pregnant women at high risk of fetal chromosomal abnormalities. This includes women of advanced maternal age, a family history of chromosomal anomalies, and those with concerning results from prenatal ultrasound or high-risk Quad Tests (2).

Amniocentesis in Thailand is primarily performed in large hospitals, including provincial hospitals, Centers of Excellence hospitals, and medical university hospitals. However, Sanpatong Hospital, a mid-level secondary hospital, only began offering this procedure in 2016 to help reduce the workload and to alleviate the strain on resources at larger institutions. This initiative aimed to increase access to this service for high-risk pregnant women, while simulta-neously reducing their travel time and financial burden. Since the start of second-trimester genetic amniocentesis at Sanpatong Hospital, however, there has been no data regarding the safety of the procedure in the hospital or the prevalence of abnormal chromosomes.

The purpose of this study was to assess the indications, complications and outcomes of second-trimester genetic amniocentesis performed in Sanpatong Hospital.

METHODS

This study was approved by the Sanpatong Hospital Research Ethics Committee (approval number: SPT-REC 001/2566). The study analyzed data collected from high-risk pregnant women with fetal chromosomal abnormalities, including women of advanced maternal age (>35 years at the estimated due date), a family history of chromosomal anomalies, those with concerning results from prenatal ultrasound or high-risk Quad Tests (abnormal level of biochemical profile of alphafetoprotein (AFP), human chorionic gonadotropin (hCG), unconjugated estriol (uE3), and inhibin A (inh A), which increase the risk of aneuploidy of trisomy 13, 18 and 21 to more than 1:250), between October 1st, 2016 and September 30th, 2022 who underwent a second-trimester genetic amniocentesis at the antenatal care unit of Sanpatong Hospital, Chiang Mai, Thailand.

The sample size for this study was calculated using the following formula:

$$n = \frac{N(Z\alpha/2)^2P(1-P)}{d^2(N-1) + (Z\alpha/2)^2P(1-P)}$$

where $Z_{\alpha/2}$ represents the 95% confidence level, P represents the prevalence of fetal chromosomal abnormalities (2.5%), N represents the total population size (451 cases), and d represents the desired level of precision (98%). The calculated sample size was 155 cases. As the population size was relatively small, the entire population was included in the study.

High-risk pregnant women were fully counseled on the benefits and risks of amniocentesis prior to the procedure. Informed consent was obtained for all procedures.

After counseling, a detailed ultrasound examination was performed using a Toshiba TUS-X100s ultrasound machine. This standard examination determined the number of fetuses, fetal viability, gestational age, amniotic fluid volume, and placental location.

The amniocentesis procedure followed standardized protocols. The abdomen was prepped with 10% povidone-iodine antiseptic, sterile drapes were applied and a 22-gauge spinal needle was inserted under realtime ultrasound guidance used a "two-person technique." One team member scanned the image while the other aspirated the amniotic fluid with three 10-cc syringes. The first syringe collected 1-2 cc, while the following syringes collected 8 cc each.

After the needle had been withdrawn, the women were shown the puncture site, the ultrasound image confirming the well-being of the fetus, and the measured amniotic fluid volume. After a 30-minute rest period, the women were discharged from the hospital if no complications had arisen. A follow-up appointment was scheduled one month later to discuss the results of the analysis with the patient and to monitor for potential complications. Additionally, the women were instructed to immediately seek medical attention at Sanpatong Hospital if any complications arose.

The collected amniotic fluid was subsequently sent to Bangkok Cytogenetics Center Co., Ltd. in Bangkok, Thailand for fetal karyotype analysis. When the cell culture was successful, the laboratory reported the fetal karyotype. If a cell culture

failed or if there was no cell growth, a QF-PCR test was performed by the Center and results were reported within a week.

This study collected data on demographic information, obstetric information, indications for amniocentesis, results of amniocentesis, complications, and pregnancy outcomes. The data are presented as counts, percentages, means, and standard deviations.

RESULTS

Our study identified 451 high-risk pregnant women who received second-trimester genetic amniocentesis at Sanpatong Hospital, Chiang Mai, Thailand, for various indications between October 1st, 2016, and September 30th, 2022. Of these women, the majority (51.3%) were aged 35-39 years. Notably, among mothers who underwent amniocentesis and had fetuses with chromosomal abnormalities, the highest proportion (35.7%) were in the 35-39 age group, followed by the 25-29 age group (28.6%). Ninety percent of the procedures were performed between 16 and 18 weeks of gestation (range 16-23 weeks) (Table 1).

Our study found the most common indication for amniocentesis was advanced maternal age, which has the same risk as high-risk serum screening (Quad test), accounting for 49.4% (223/451) of cases. Abnormal ultrasound findings were the second most common indication at 1.2% (5/451). Some cases had two indications for amniocentesis, including both advanced maternal age and high-risk serum screening (Quad test), accounting for 19.1% (86/451) of cases. The most common indication found to be associated with chromosomal abnormalities was high-risk serum screening (Quad test), accounting for 64.3% (9/14) of cases followed by advanced maternal age at 28.6% (4/14) and ultrasound abnormalities at 7.1% (1/14) (Table 2).

In our study the success rate of cell culture from amniocentesis was 98.9% (446/451). Five cases (1.3%) failed to culture cells, but all were found to have no chromosomal abnormalities of chromosomes 13, 18, 21, X, and Y by QF-PCR. The prevalence of fetal chromosomal abnormalities

Table 1. Maternal characteristics

Parameter	Amniocentesis		Abnormal chromosome	
	Number (n=451)	Percent	Number (n=14)	Percent
Maternal age (years)				
< 20	10	2.2	1	7.1
20-24	25	5.5	0	0.0
25-29	56	12.4	4	28.6
30-34	55	12.2	1	7.1
35-39	231	51.3	5	35.7
40-44	69	15.3	3	21.5
≥ 45	5	1.1	0	0.0
Gestational age (weeks)				
< 16	0	0.0	0	0.0
16-18	406	90.0	13	92.9
19-21	44	9.8	1	7.1
> 21	1	0.2	0	0.0

Table 2. Indication of second trimester genetic amniocentesis

Indications	Amniocentesis (n=451)	Percentage	Abnormal chromosome (n=14)	Percentage
Advanced maternal age	223	49.4	4	28.6
Positive serum screening	223	49.4	9	64.3
- Age < 35 years	137	30.4	5	35.7
- Age ≥ 35 years	86	19.1	4	28.6
Anomaly from ultrasound	5	1.2	1	7.1

Table 3. Assessment of clinical data

Indications	Abnormal chromosome (n=14)	Percentage
Culture result		
- Successful	446	98.9
- Failure	5	1.1
Chromosomal result		
- Normal	432	95.8
- Abnormal	14	3.1
- Failed culture result	5	1.1

was 3.1% (14/451) (Table 3).

Among the 14 cases of abnormal chromosomes, trisomy 21 was the most common, accounting for 1.3% (6/446). The remaining cases comprised various abnormalities, including translocation (1.0%, 4/446) and sex chromosomal abnormalities (0.4%, 2/446) (Table 4).

All complications were identified within the 30-day follow-up period following the procedure. Minor complications, including pelvic pain (0.6%) and placental hematoma (0.2%), were subsequently managed effectively with conservative treatment (Table 5). Notably, no miscarriages were attributed to the procedure within this 30-day period.

The pregnancy outcomes of 203 cases (45.5%) with normal fetal chromosomes were followed, including 25 cases (12.3%) of preterm delivery. Among the preterm deliveries, 2 cases (1%) were extremely preterm deliveries, 4 cases (2%) were very preterm, 3 cases (1.5%) were moderately preterm, 16 cases (8%) were late preterm, and the remaining 178 cases (87.7%) were normal term (Table 6).

DISCUSSION

Second-trimester genetic amniocentesis is a standard procedure that involves withdrawal of amniotic fluid from the amniotic sac for cell culture and diagnosis of fetal chromosome abnormality. Previously, amniocentesis was performed by a single operator using an ultrasound guide to insert a needle and then place an ultrasound probe to aspirate the amniotic fluid. However, in this study, a two-person technique was used, with realtime ultrasound guidance and amniotic fluid aspiration. This technique was introduced to assess the indications, complications, and outcomes of second-trimester genetic amniocentesis performed at Sanpatong Hospital between 2016 and 2022. The results provide valuable insights into the safety and efficacy of

Table 4. Assessment of the chromosomal results

Results	Chromosomal results (n=446)	Percentage
Normal chromosome	432	96.9
- 46 XX	212	47.5
- 46 XY	220	49.4
Abnormal chromosome	14	3.1
Numerical abnormalities		
- Trisomy 18	2	0.4
- Trisomy 21	6	1.3
Sex chromosome abnormalities		
- 45 X	2	0.4
Structural abnormalities		
- 46 XY, inv(9)(qh)	2	0.4
- 46 XY,qh+	1	0.3
- 46 XX,t(1;4)(p13;q12)	1	0.3

Table 5. Complication of procedure within 30 days (N=4)

Complication	Number	Details	Outcome	Percentage
Placental hematoma	1	Intra-procedure	Continued to term	0.2
Pelvic pain	1	1 day after procedure	Continued to term	0.2
Pelvic pain	2	3 days after procedure	Continued to term	0.4

Table 6. Assessment of the chromosomal results

Outcome	Number	Percentage
Preterm delivery	25	12.3
- Extremely preterm (GA < 28 weeks)	2	1.0
- Very preterm (GA 28-31 weeks)	4	2.0
- Moderately preterm (GA 32-33 weeks)	3	1.4
- Late preterm (GA 34-36 weeks)	16	7.9
Full term delivery	178	87.7

this procedure in a mid-level secondary hospital setting. Most of the women who underwent amniocentesis were aged 35-39 years, with the most common gestational age for the procedure being 16-18 weeks. This aligns with existing literature (8-10). Notably, following the implementation of the Quad test policy at Sanpatong Hospital, the study found a 30.4% increase in the number of women under 35 years of age who were identified as high-risk by Quad test and who subsequently underwent amniocentesis. Among this group, 3.7% (5/137) of the fetuses were found to have chromosome abnormalities. Advanced maternal age and high-risk serum screening results were the most common indications for amniocentesis, followed by abnormal ultrasound findings. These findings likely reflect both the Quad test policy and the concerns of high-risk pregnancy women regarding the potential risks of amniocentesis. These findings diverge from previous studies (8-10) which have reported advance maternal age was the most common indication, but in our studies suggests a change practice for high-risk serum screening was the most common indication.

Our study had a high success rate of cell culture (98.9%), with only 1.1% of cases failing to culture cells, lower than other studies (8-10). Notably, studies in Western countries have reported culture failure rates as low as 0.44% (12). Factors potentially impacting cell culture success include maternal blood contamination, bacterial contamination, insufficient fluid volume, discolored amniotic fluid, and contaminated culture media (13). While some authors have suggested an association between amniotic fluid culture failure and fetal chromosomal abnormalities, we were unable to confirm this relationship in our study. All cases with documented procedures that resulted in amniotic fluid culture failure in this study exhibited normal fetal chromosomes.

The prevalence of fetal chromosomal abnormalities in this study was 3.1%, with trisomy 21 identified as the most common aneuploidy (1.3%). This observed rate exceeds those reported in other studies (8-10), potentially reflecting Sanpatong Hospital's role as a referral center serving the southern region of Chiang Mai province.

This study found no miscarriages attributable to the procedure within 30 days. One percent of women experienced a post-procedural abortion, a rate consistent with existing literature (8-10). Minor complications occurred in a small fraction of cases (0.6% pelvic pain, 0.2% placental hematoma) and were effectively managed conservatively. Previous research has identified factors potential contributing to amniocentesis complications, including needle gauge > 20, placental penetration and more than two needle insertions (14). Placental hematoma has been reported in up to 1.3% of cases (15, 16).

A key strength of the study was that it was the first to report on second-trimester genetic amniocentesis performed in a mid-level secondary hospital. The study found that standardized protocols were used for counseling, ultrasound examination, and the amniocentesis procedure. Additionally, data were collected on various aspects of the procedure, including indications, complications, and pregnancy outcomes.

A limitation of this study was the ability to follow up on only 42.2% of pregnancy out-comes. This was due to the fact that Sanpatong Hospital serves primarily high-risk pregnancy women who undergo amniocentesis. When their test result is normal, these women return to their home hospitals for antenatal care and delivery. This could have resulted in inaccurate data for miscarriages, preterm birth, or term birth.

CONCLUSIONS

This study demonstrated that second trimester genetic amniocentesis performed at Sanpatong Hospital, a mid-level secondary hospital, over a six-year period resulted in no fetal losses.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

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Original Article **Open Access**

Clinical Characteristics and Outcomes of Primary Vitreoretinal Lymphoma in Northern Thailand

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ABSTRACT

OBJECTIVE This study aims to describe clinical characteristics and outcomes after treatment of primary vitreoretinal lymphoma (PVRL).

METHODS Fifteen patients with a proven diagnosis of PVRL by histology, cytology and/or flow cytometry were analyzed.

RESULTS The median age of the 15 patients was 59 years (range 41-71). Median follow-up time was 37 months (IQR 22.5-80) (range 4-106). Ophthalmic presentations of 25 eyes included vitritis (72%), chorioretinal infiltrations (60%), and retinal vasculitis (20%). Bilateral involvement was observed in 10 patients at presentation and in 4 patients during follow up. Ten patients (67%) developed brain involvement after ocular presentation with a median time of 22.5 months (range 2-84). Treatment modalities were included: 1) isolated intravitreal (IVT) methotrexate (6/15 patients; 40%) with a median number of injections of 4 (IQR 1,6) (range 1-16) 2) combined with IVT methotrexate and/or rituximab and systemic chemotherapy and/or radiation (8/15; 53%) with a median of 6 injections (IQR 1,11) (range 1-16) and 3) systemic chemotherapy alone (1/15; 7%). Whole brain radiotherapy (WBRT) was performed in 10 of 15 patients (67%). Among the 6 patients who received isolated IVT methotrexate, 3 patients had complete remission (3/6; 50%), one died at 96 months after treatment, and one was lost to follow up after a single injection. Nine of 15 patients who received systemic chemotherapy with or without IVT chemotherapy and/or WBRT had complete remission (8/9; 89%).

CONCLUSIONS Vitritis and chorioretinal infiltrations were the main ocular presentations of PVRL. Two-thirds of the patients developed brain involvement which resolved after treatment. Systemic chemotherapy tends to provide a higher rate of complete remission compared to local therapy alone.

KEYWORDS primary vitreoretinal lymphoma, intraocular lymphoma, intravitreal methotrexate, intravitreal rituximab, Thailand

INTRODUCTION

Primary vitreoretinal lymphoma (PVRL) is an uncommon intraocular malignancy which is classified as a diffuse large B-cell lymphoma in most cases. Currently, PVRL is categorized as a subtype of primary central nervous system lymphoma (PCNSL). Its incidence was reported to be 0.28% per 100,000 persons per year in immuno-

competent patients and to be more prevalent in immunocompromised patients (1). There are limited data regarding the worldwide incidence of PVRL due to its rarity, but most studies suggest its prevalence is increasing (1-4).

Typically, PVRL occurs in elderly patients and its clinical manifestations can resemble intraocular inflammation. For that reason, PVRL also carries the name "masquerade syndrome". This intraocular inflammation presentation may lead to delayed or missed diagnosis (5). Several tools have been used to help in the diagnosis of PVRL including ocular and brain imaging and laboratory tests such as ocular fluid or tissue samples for cytology, histology, immunocytochemistry for CD20, flow cytometry, biochemical analysis (interleukin-10/interleukin-6 ratio) and polymerase chain reaction for gene mutations such as myeloid differentiation primary response 88 (MYD88) mutation (6).

PVRL is frequently accompanied by CNS involvement and has a poor prognosis. Current treatment includes various approaches. Due to its rarity, there is no consensus regarding the best treatment strategy. Treatment modalities include ocular chemotherapy as well as combined ocular and systemic chemotherapy with or without radiotherapy of CNS, eyes, or both. More recently, autologous stem cell transplantation is also being used (6). The choice of modalities depends on the extent of the disease, age and baseline status of the patient (7). Unfortunately, the survival rate of PVRL is approximately 34 to 44 months (8).

We conducted this study to assess clinical characteristics and final outcomes of patients with a proven diagnosis of PVRL.

METHODS

The study was approved by the Institutional Ethics Committee of faculty of medicine, Chiang Mai University. The study included 15 patients with proven PVRL from the Department of Ophthalmology, Chiang Mai University Hospital from 2008 through 2021. All patients underwent pars plana vitrectomy either with or without retinal biopsy and had positive results of tests for intraocular lymphoma by histology, cytology and/or flow cytometry. The patients' medical records were reviewed for demographic data including age, gender, underlying diseases, immune status, and laterality. Clinical characteristics, including best corrected visual acuity (VA) at first visit and final visit, ocular symptoms and signs, ocular imaging (i.e., fundus photography, optical coherence tomography (OCT), fundus fluorescein angiography (FA)), non-ocular involvement, time to diagnosis of PVRL, cell morphology and immunocytochemistry results were collected.

Treatment modalities were categorized as isolated intravitreal (IVT) with methotrexate (0.4 mg/0.1 mL) combined IVT with methotrexate and/or rituximab (1 mg/0.1 mL) and systemic chemotherapy with or without brain/ocular radiation. Regarding the systemic chemotherapy regimen, the DeAngelis protocol (methotrexate, leucovorin, vincristine, procarbazine and dexamethasone) was used most frequently. The CHOP regimen (cyclophosphamide, doxorubicin, vincristine and prednisolone) still has a role in treatment of lymphoma, though it was given to only one patient. Final outcomes after treatment, including VA, brain involvement, and progression of disease were analyzed. Complete remission was defined as no recurrence of ocular lesions or systemic disease during the follow-up period.

Statistical analysis

Categorical data is shown as percentages. Numerical data are presented as a median (interquartile range; IQR). Survival time is demonstrated by Kaplan-Meier estimates curve. The duration of remission is calculated from the first date on which patients had stable activity of the disease to the date of the last follow-up, relapse, progression, or death from any cause.

RESULTS

Demographic data are presented in Table 1.1 and 1.2. The median age of the 15 patients was 59 years (range 41-71) with more females affected than males (ratio 2:1). The median time to diagnosis was 8 months (range 1-14) counted from the onset of the first symptoms. Bilateral involvement was observed in 10 patients (67%). HIV infection was present in 2 patients (13%). Diabetes mellitus and hypertension were mostly found as underlying diseases, and all were under control.

The most common symptoms at the first presentation were blurred vision (94%) and floaters (40%). Initial VA varied from 6/6 to light perception. The median (IQR) of VA (Log MAR) in the affected eye was 0.5 (0.2-1.25) (range 0.2-3.0).

The most common ocular findings were vitritis (72%; 18/25 eyes), anterior uveitis (60%; 15/25 eyes) and retinal lesions (60%; 15/25 eyes). Retinal vasculitis was observed in 20% (5/25 eyes). Vitritis, chorioretinal lesion and retinal vasculitis are shown in Figure 1A-C.

OCT of 13 patients (16 eyes) were able to be analyzed. Subretinal infiltration and sub-RPE deposits were the most frequently observed (50%; 8/16 eyes) (Figure 1D). Other findings included intraretinal infiltration (46%) and focal disruption of photoreceptors (25%). FA was performed in 3 patients of whom one exhibited hypo and hyper fluorescence with a granular pattern consistent with a leopard spot pattern with late leakage (Figure 1E).

Most diagnostic samples were obtained from vitreous samples (10/15 patients; 67%). Three patients (20%) were diagnosed with lymphoma based on brain tissue biopsy. Two of eleven patients (18%) who underwent retinal biopsy were recognized as having intraocular lymphoma from retinal tissue biopsy results. Eight patients were labeled as B-cell type (53%), three patients as T-cell type (20%) and the rest were labeled as atypical lymphocytes (Table 1.1).

Cerebrospinal fluid examination was performed in 10 patients (67%) and was positive in only one patient (10%). All patients had CT or MRI brain imaging. One patient had brain involvement at the time of the diagnosis.

The treatment modalities are shown in Table 1.2. Six of 15 patients (40%) received only IVT chemotherapy, most of whom received methotrexate. The median number of injections per eye was 4 (IQR 1,6) (range 1-16).

IVT combined with systemic chemotherapy was provided in 8 patients (53%). Rituximab was used in combination with methotrexate in 2 patients (13%) with concurrent systemic chemotherapy. One patient (7%) underwent systemic chemotherapy combined with ocular radiation.

IVT chemotherapy regimens used in our center were methotrexate and/or rituximab. The median number of IVT methotrexate and rituximab injections was 6 (IQR 1,10.5) (range 1-16) and 2 (IQR 1,6) (range 0-6), respectively. DeAngelis regimen was used primarily as systemic chemotherapy. Whole brain radiotherapy was given to ten patients (67%). The IVT, systemic chemotherapy and brain radiotherapy were the most frequently used regimen (7 patients; 67%).

Ten of 15 patients (67%) developed brain involvement later despite receiving the treatment. The duration of the interval between ocular lymphoma and brain involvement ranged from 2 months to 84 months with median time of 22.5 months.

Among the 6 patients who received isolated IVT methotrexate, 3 patients had complete remission (3/6; 50%), one died at 96 months after treatment, and one was lost to follow-up after a single injection. Nine of 15 patients who received systemic chemotherapy with or without IVT chemotherapy and/or WBRT had complete remission (8/9; 89%).

After completing the treatment, eleven patients (73%) had complete remission. Three patients (25%) were unilaterally blind. One patient was lost follow-up during the study. One patient was in remission for 8 years but died after a relapse of the disease.

VA outcomes varied from 6/6 to light perception. The median VA outcomes in the right and left eye were 0.65 (0.225-2.375) and 0.2 (0.05-0.575), respectively. The median VA outcomes of all 25 eyes with PVRL without laterality was 0.6 (0.2-2.4). The median period of progression-free survival was 13 months (IQR 4-61) (range 0-96) and the median follow-up time was 37 months (IQR 22.5-80) (range 4-106). (Figure 2)

A summary of demographic data, laboratory results and treatment modalities are shown in Table 2.

DISCUSSION

Our study demonstrated that vitritis and chorioretinal infiltrations with OCT of subretinal infiltration and sub-RPE deposits were the main ocular presentations of PVRL and affected more females than males. Several studies have reported that the average age of PVRL patients to be in the range of 60 years, which corresponds with our study population (3,4,9). There was no difference in the average age of patients in this study compared to other studies of Asian subjects (10).

Kimura et al. (10) and Kim et al. (11) found the average intervals from initial evaluation to diagnosis were 10.6 and 11 months, respectively, which is comparable to the present study (8 months). The delay in diagnosis could be due to the variety of ocular manifestations of PVRL which can mas-

Figure 1. A. Fundus photography of primary intraocular lymphoma showed vitreous haze with yellowish subretinal infiltration, B. Fundus photography of primary intraocular lymphoma demonstrated creamy yellowish subretinal infiltration (Leopard spot), C. Fundus photography of primary intraocular lymphoma demonstrated retinal vasculitis, D. Optical coherence tomography demonstrated irregular retinal pigment epithelial contour with subretinal and intraretinal hyperreflectivity deposit and photoreceptor disruption, E. Fluorescein angiography showed early hyper-hypo fluorescent spots with late hyperfluorescent staining.

Table 1.1 Clinical characteristics and laboratory results of 15 primary vitreoretinal lymphoma patients

Patient	Gender age (year)	Immune status	Laterality at onset	Underlying diseases	Ocular signs	Initial VA (OD, OS)	Final VA (OD, OS)	Positive samples	Cell type	Cytology result	Flow cytometry result
1	Male 68 Y	Immuno-competent	Bilateral	HT	Retinis and vitritis	6/18, 6/36	HM, 6/9	Vitreous	Atypical lymphocytes	Negative	Positive (CD20, CD45)
2	Female 61 Y	Immuno-competent	Unilateral	DM	Anterior uveitis and vitritis	Fc 2 ft (OS)	6/18 (OS)	Vitreous	Atypical lymphocytes	Negative	Positive (CD3, CD4, CD5, CD7, CD8)
3	Female 47 Y	Immuno-competent	Unilateral	None	Anterior uveitis, retinitis, vitritis and vasculitis	HM (OD)	NPL (OD)	Vitreous	Atypical lymphocytes	Negative	Positive (CD3, CD4, CD8)
4	Female 65 Y	Immuno-competent	Bilateral	None	Anterior uveitis, retinitis, vitritis and vasculitis	PL, 6/60	NPL, NPL	Vitreous	T cell	Positive (Large lymphoid cells)	Not done
5	Female 71 Y	Immuno-competent	Bilateral	HT, DM	Anterior uveitis, retinitis, vitritis and vasculitis	6/18, HM	6/36, NPL	Vitreous	B cell	Positive (Large lymphoid cells)	Positive (CD3, CD20)
6	Female 60 Y	Immuno-competent	Bilateral	None	Vitritis	HM, 6/9	HM, 6/9	Vitreous	B cell	Positive (Atypical lymphoid cells)	Positive (CD20)
7	Male 54 Y	Immuno-competent	Unilateral	HT, DM	Anterior uveitis, retinitis and vitritis	6/24 (OS)	6/24 (OS)	Vitreous	Atypical lymphocytes	Positive (Atypical lymphoid cells)	Negative
8	Male 61 Y	Immuno-competent	Unilateral	DM	Anterior uveitis and vitritis	Fc 1 ft (OD)	6/18 (OD)	Vitreous	T cell	Positive (Atypical lymphoid cells)	Positive (CD3, CD10, CD19)
9	Female 56 Y	Immuno-competent	Unilateral	None	Anterior uveitis, vitritis and vasculitis	Fc 3 ft (OD)	HM (OD)	Retinal tissue	B cell	Positive (Atypical lymphoid cells)	Not done
10	Female 43 Y	Immuno-compromised (HIV)	Bilateral	COPD	Retinitis, vitritis and vasculitis	6/9, 6/24	6/24, 6/24	Vitreous and retinal tissue	B cell	Positive (Atypical lymphoid cells)	Not done
11	Male 59 Y	Immuno-compromised (HIV)	Bilateral	None	Anterior uveitis and vitritis	6/9, 6/9	6/9, 6/9	Vitreous	T cell	Positive (Atypical lymphoid cells)	Not done
12	Male 51 Y	Immuno-competent	Bilateral	None	Retinitis and vitritis	6/6, 6/9	6/6, 6/9	Brain	B cell	Negative	Not done
13	Female 52 Y	Immuno-competent	Bilateral	None	Retinitis and vitritis	HM, HM	6/60, HM	Brain	B cell	Negative	Not done
14	Female 41 Y	Immuno-competent	Bilateral	None	Vitritis and anterior uveitis	HM, 6/9	6/9, 6/6	Brain	B cell	Negative	Negative
15	Female 60 Y	Immuno-competent	Bilateral	None	Anterior uveitis, retinitis and vitritis	HM, HM	NPL, PL	CSF	B cell	Negative	Negative

Table 1.2 Treatment modalities, brain involvement and outcomes of 15 primary vitreoretinal lymphoma patients

Patient	Gender age (year)	Initial VA (OD, OS)	Final VA (OD, OS)	Cell type	Ocular treatment*	Systemic treatment**	WBRT	Systemic involvement	Preceding time of primary organ	Side effects of treatment	Outcomes at 1 year after diagnosis
1	Male 68 Y	6/18,6/36	HM,6/9	Atypical lymphocytes	IVT Methotrexate	None	None	Brain	84 months	None	Perish
2	Female 61 Y	Fc 2 ft (OS)	6/18(OS)	Atypical lymphocytes	IVT Methotrexate	None	Yes	Brain	15 months	None	Remission
3	Female 47 Y	HM (OD)	NPL(OD)	Atypical lymphocytes	IVT Methotrexate	None	None	None	None	None	Remission
4	Female 65 Y	PL,6/60	NPL,NPL	T cell	IVT Methotrexate	De-Angelis	Yes	Brain	4 months	Headache	Active disease
5	Female 71 Y	6/18, HM	6/36,NPL	B cell	IVT Methotrexate and IVT rituximab	De-Angelis	Yes	None	None	Skin irritation	Remission
6	Female 60 Y	HM,6/9	HM,6/9	B cell	Ocular radiation	De-Angelis	Yes	Brain	11 months	None	Remission
7	Male 54 Y	6/24 (OS)	6/24(OS)	Atypical lymphocytes	IVT Methotrexate	De-Angelis	None	None	None	None	Remission
8	Male 61 Y	Fc 1 ft (OD)	6/18(OD)	T cell	IVT Methotrexate	None	None	None	None	None	Active disease
9	Female 56 Y	Fc 3 ft (OD)	HM (OD)	B cell	IVT Methotrexate	CHOP	Yes	Brain	2 months	Nausea	Remission
10	Female 43 Y	6/9,6/24	6/24,6/24	B cell	IVT Methotrexate and IVT rituximab	De-Angelis	Yes	Brain	6 months	None	Remission
11	Male 59 Y	6/9,6/9	6/9,6/9	T cell	IVT Methotrexate	None	None	None	None	None	Loss follow up
12	Male 51 Y	6/6,6/9	6/6,6/9	B cell	IVT Methotrexate	De-Angelis	Yes	Brain	5 months	None	Remission
13	Female 52 Y	HM, HM	6/60, HM	B cell	IVT Methotrexate	De-Angelis	Yes	Brain	12 months	Nausea	Remission
14	Female 41 Y	HM,6/9	6/9,6/6	B cell	IVT Methotrexate	De-Angelis	Yes	Brain	1 month	Nausea	Remission
15	Female 60 Y	HM, HM	NPL, PL	B cell	IVT Methotrexate	None	Yes	Brain	1 month	Nausea	Remission

*Intravitreal (IVT) methotrexate (0.4 mg/0.1 mL) with frequency of weekly injection; IVT rituximab (1 mg/0.1 mL) with frequency of one injection in two to four weeks until the patients received systemic chemotherapy with or without brain radiation

**Systemic treatment composed of De Angelis Regimen (methotrexate, leucovorin, vincristine, procarbazine and dexamethasone) or CHOP regimen (Cyclophosphamide, Doxorubicin, Vincristine and Prednisolone)

HT, hypertension DM: Diabetes mellitus; Fc, finger count; Ft, foot; HM, hand motion; PL, light perception; NPL, no light perception; CSF, cerebrospinal fluid; HIV, human immunodeficiency virus; WBRT, whole brain radiation therapy

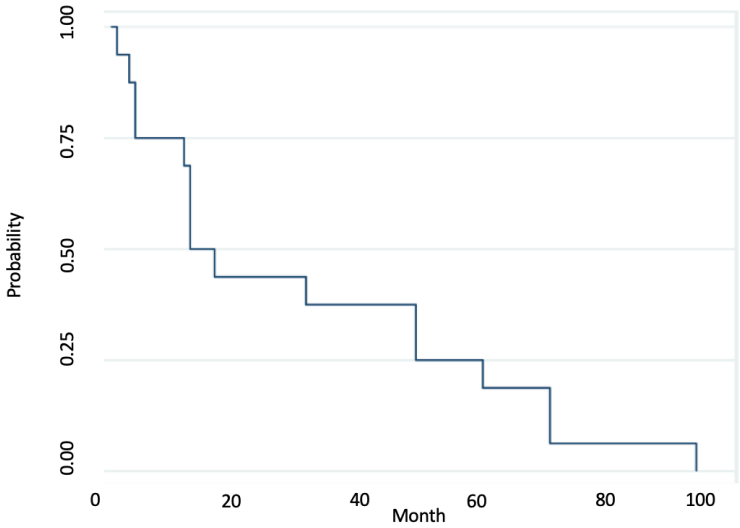

Figure 2. Kaplan-Meier estimate curve showing progression-free survival of 15 primary vitreoretinal lymphoma patients.

Table 2. Summarized table of demographic data, laboratory results and treatment modalities

Male to female ratio	Average age (year)	HIV infection	Bilateral involvement at onset	B cell to T cell ratio	Positive cytology	Positive flow cytometry	Ocular chemotherapy	Systemic chemo-therapy	Radiation (ocular/whole brain)
1:2 (5:10)	57±8.57	2/15	Unilateral: 5/15 Bilateral: 10/15	8:3	8/15	6/9	IVT MTX: 12/15 IVT MTX+RTX: 2/15 None: 1/15	9/15	10/15 (ocular RT 1, WBRT 9)

IVT, intravitreal; MTX, Methotrexate (0.4 mg/0.1 mL); RTX, Rituximab (1 mg/0.1 mL)

querade as multiple ocular conditions. Additionally, achieving a correct diagnosis of PVRL requires the use of multimodal technologies that are usually available only in referral centers.

Previous studies (3, 4, 9, 10) have found that B-cell lymphoma is the most common type in PVRL which is similar to the present study. All three patients in this study who had T-cell types shared common features of anterior uveitis and vitritis. OCT imaging depicted intraretinal infiltration in 2 of 3 patients. People with B-cell lymphomas often have a better prognosis than those with T-cell lymphomas (2). In this study, Two of 3 patients with T-cell lymphoma still had an active disease after one year from the diagnosis.

In OCT imaging, outer retinal and subretinal abnormalities were the most frequently detected which is comparable to other studies (12, 13). At initial examination, some images could not be obtained due to severe vitritis. For that reason, the severity of abnormalities observed might be lower if OCT were performed after partial treatment.

Presently, there is no consensus regarding the treatment regimen for PVRL or PCNSL (14). Multidisciplinary approaches which include hematologists and radiologists are needed to create a treatment plan. Methotrexate is still a main protagonist used for this disease in both ocular and systemic involvements. According to the International PCNSL Collaborative Group Symposium (14), local therapies, e.g., IVT chemotherapy and ocular radiation, were selected for localized PVRL despite bilateral involvement, while systemic chemotherapy was omitted in cases of CNS involvement to prevent excessive side effects. In our study, IVT methotrexate was used in most cases, both isolated and combined with systemic chemotherapy. This study found that isolated IVT chemotherapy had a lower remission rate: there was only one patient who had a complete remission for 8 years after CNS development. Patients who had systemic chemotherapy tend to have a higher rate of complete remission when compared to only local therapy.

Due to the aggressive course of this disease, PVRL patients still had only a fair prognosis even though extensive treatment, including combined local and systemic therapy, was provided. Riemens

et al. (8) reported that the median overall survival of PVRL patients without CNS involvement was 44 months and 34 months in those with CNS involvement. The progression-free survival in our study (13 months (IQR 4-61)) was significantly less than Riemens et al. (8) This might be affected by our patients being referred to another hospital after complete remission, leading to shorter follow - up time.

Our study had several limitations, including a limited number of patients and a shorter follow time than other studies (8, 10, 11). The vitreous cytology examination is a useful investigation for definite diagnosis of PVRL. Nevertheless, malignant cells are quite scarce and prone to degenerate easily (9, 15). This barrier decreases the chance of vitreous cytology to diagnose PVRL.

CONCLUSIONS

This study demonstrated that vitritis and chorioretinal infiltrations are the main ocular presentations of PVRL. More than half of the patients developed brain involvement, but that can be resolved after treatment. Systemic chemotherapy improved the rate of complete remission compared to local therapy alone.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

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Hippocampal Avoidance Prophylactic Cranial Irradiation using Helical Tomotherapy in Small Cell Lung Cancer

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ABSTRACT

OBJECTIVE Prophylactic cranial irradiation (PCI) is a standard treatment for small cell lung cancer (SCLC). Reduced radiation doses at the hippocampal region during PCI might protect against neurocognitive decline after radiotherapy (RT). The purpose of this study is to report the outcome of hippocampal avoidance PCI (HA-PCI) in our center.

METHODS After the initial treatment of SCLC, patients whose radiographic data confirmed the absence of intracranial metastasis and controlled of the primary disease received HA-PCI. Thai Mental State Examination (TMSE) and bilateral hippocampal volumes were recorded and analyzed using the Wilcoxon Singed Rank test to compare baseline and two time points (3 and 6 months). The two-year overall survival (OS) and brain control rates were estimated using the Kaplan Meier method.

RESULTS Between 2018 and 2021, a total of 10 patients were included in the analysis. The median TMSE and bilateral hippocampal volumes showed no statistically significant difference between baseline and 3 and 6 months. Two-year OS and brain control rates were 78.8% and 71.4%, respectively. Three patients developed intracranial relapses after HA-PCI which were located outside the hippocampal region.

CONCLUSIONS HA-PCI did not increase intracranial relapse in this study. HA-PCI should be considered as a treatment option which can potentially protect neurocognitive functions.

KEYWORDS hippocampal avoidance, prophylactic cranial irradiation, small cell lung cancer

INTRODUCTION

Small cell lung cancer (SCLC) is a subtype of pulmonary cancer with a high propensity for spreading to the brain. The incidence of intracranial metastases is approximately 10%-14% at the time of diagnosis and 40%-50% during progression of the disease (1, 2). SCLC is classified as limited disease (LD) and extensive disease (ED) following the Veteran's Administration Lung Cancer Study Group (3). Systemic chemotherapy and thoracic radiation therapy (RT) constitute the primary treatment for SCLC. In both LD and ED, patients who did not experience disease progression were further treated with prophylactic cranial irradiation (PCI) (4, 5). However, PCI can potentially affect neurocognitive function from radiation painting to the entire brain. Higher radiation exposures to the whole brain have been found to be directly

correlated with more neurological deterioration (6, 7). Therefore, the standard PCI dose is 25 Gy in 10 fractions (Fx). Another strategy to reduce neurocognitive deficits is to minimize the radiation dose along bilateral hippocampal regions. This technique is typically applied to patients who require whole brain irradiation to diminish multiple brain metastasis (8). According to previously published preclinical data, memory-specific neural stem cell components reside in the hippocampal region, the subgranular zone (SGZ) of the dentate gyrus, and the subventricular zone (SVZ) which is located on the lateral aspect of the lateral ventricle (9, 10). These neuro-progenitor stem are extremely radiosensitive and are easily destroyed by radiation (11, 12). The outcome of the Radiation Therapy Oncology Group (RTOG) 0933 study indicated that hippocampal-avoidance whole brain radiotherapy (HA-WBRT) with a prescribed radiation dose of 30 Gy in 10 Fx reduced memory impairment in patients with multiple brain metastases (8). Hippocampal-avoidance zones (HAZ) are defined as 5-mm isotropic expansions from bilateral hippocampi. Radiation dose restrictions in these regions are 16 Gy for the maximum dose (Dmax) and 9 Gy for 100% of the HAZ volume (D100%). The hippocampal metastatic rate following HA-WBRT was 4.5%, which is lower than the estimated rate of 8.6%. However, RTOG 0933 did not include patients with primary SCLC in the study and there were few data regarding the prevalence of hippocampal metastases in SCLC. Some studies have reported finding 5.1% of de novo brain metastases in the hippocampi and that 5% of patients who received WBRT experienced a relapse in the hippocampi (13). Consequently, a lower radiation dosage to the bilateral hippocampi may be safe for SCLC patients requiring PCI as part of the standard treatment. Moreover, a reduced radiation dose to these regions may reduce the neurocognitive deficit induced by radiation.

This study aimed to report the outcome after hippocampal-avoidance PCI (HA-PCI) for SCLC patients who were treated at our center using a Helical Tomotherapy (HT) machine.

METHODS

This study selected patients who fulfilled the following inclusion criteria: Age 18 to 80 years; Thai nationality; Completion of the Thai Mental State Examination (TMSE); Eastern Cooperative Oncology Group (ECOG) performance status 0-2 (14); Histopathological diagnosis from either lung or lymph nodes confirmed as SCLC; No progression of primary tumor after chemotherapy and thoracic RT for LD-SCLC; No progression of pri mary tumor and/or extra-cranial metastases after standard systemic treatment for ED-SCLC; No evidence of intracranial dissemination on magnetic resonance imaging (MRI) before HA-PCI. Patients who had a history of cranial irradiation were excluded from this study.

After completing the standard initial treatment and confirmation of the absence of intracranial dissemination from MRI findings, patients began the radiation procedure for HA-PCI. Patients were placed in a supine position and immobilized by a thermoplastic mask. Computed-tomography simulation (CT-SIM) from the vertex to level 5 of the cervical spine without contrast media was applied using a 2-mm slice thickness. The entire brain volume, excluding the HAZ, was defined as the clinical target volume (CTV). The planning treatment volume (PTV) was expanded by 3-mm from the CTV. Cranial MRI was registered in the CT-SIM dataset in order to delineate bilateral hippocampi and other critical structures, including bilateral orbits, lenses, optic apparatus, brainstem, and pituitary gland. The hippocampal organs were contoured in accordance with RTOG 0933 recommendations (8). All patients in this study received radiation planning and delivery by HT.

Radiation dose prescriptions and hippocampal dosage limitations were as follows:

Target: The whole volume of the brain exclusive of HAZ received a radiation dose of 25 Gy in 10 Fx. The prescription dose of 25 Gy covered at least 95% of PTV, Maximal dose to 2% of PTV (D2%) was limited to 31.3 Gy, and Minimum dose to 98% of PTV (D98%) was at least 20.8 Gy.

Hippocampi: Dose limitation to hippocampi was prescribed at HAZ identified by 5-mm isotropic expansion from hippocampal contouring. D100% did not exceed 10 Gy whereas D2% was less than 18 Gy.

HA-PCI was administered within 2-4 weeks after enrolling in this study. In the first year after HA-PCI completion, regular brain MRIs with contrast were evaluated every three months, and in the second year, every four months. TMSE, a

national learning test, was used to assess cognitive functions in the Thai population (15). This test evaluated memory, attention, language, executive, and visuospatial abilities. It has been extensively used in Thailand to screen for cognitive impairment and dementia, with a suggested cut-off point 23 out of 30. Patients who had a TMSE score below 23 were considered to have cognitive impairment. TMSE was tested before starting HA-PCI and during the follow-up period of MRI surveillance.

Sample size

The sample size of this pilot study was 25 patients (16), and to accommodate an estimated drop-out rate of 20%, the desired sample size in this study was set at 30 patients.

Statistical analysis

Baseline characteristics of patients, including sex, age, stage of SCLC, baseline TMSE score, and hippocampal volume, were described individually. The two-year brain control rate and overall survival (OS) rate were estimated using the Kaplan Meier method. The median scores of cognitive functions and bilateral hippocampal volumes with interquartile range (IQR) were calculated and compared between two different time points (at 3 and 6 months after completion of RT) using the Wilcoxon Signed Rank test and a statistically significance level of 5%. All analyses were performed using Stata 16.0 (Stata Corp LP, College Station, TX, USA).

Ethical approval

Ethical approval was granted by the Institutional Research Board (Grant no. 368/2561)

Clinical trial registration number

Thai Clinical Trial Registry (TCTR) ID No. TCTR20200725001.

RESULTS

From 2018 to 2021, eighteen patients with SCLC who underwent chemotherapy were referred to our department. Twelve patients had LD-SCLC, while the remaining six patients had ED-SCLC. However, four patients, two with LD-SCLC and two with ED-SCLC, were excluded from the investigation due to brain metastases detected on MRI screening. Two patients declined to participate in the protocol, and two patients died prior to CT simulation. A total of 10 patients (6 with LD- and 4 with ED-SCLC) were included in the analysis. The median age was 60 years. The median TMSE score at baseline was 23 points. The median hippocampal volumes were 2.3 cc on the right side and 2.1 cc on the left side. Patients' characteristics are presented in Table 1. Memantine was not administered to all patients in conjunction with HA-PCI due to its non-reimbursement under national universal coverage. All patients received HA-PCI in accordance with the protocol. Information regarding the dose-volume histogram (DVH) of bilateral hippocampi for each patient is shown in Table 2. After complete HA-PCI at 3 and 6 months, TMSE and bilateral hippocampal volumes showed no statistically significant difference when compared to baseline values, as shown in Table 3. No patients experienced severe acute toxicity, and none were administered steroids.

The median follow-up time was 27.8 months (IQR = 22.0-35.3 months). The two-year OS and brain control rates were 78.8% and 71.4%, respectively,

Table 1. Patients' characteristics

Patient No.	Sex	Age (year)	SCLC stage (LD or ED)	Baseline TMSE score	Hippocampal volume (cc)	
					Left	Right
1	Female	60	ED	20	2.7	2.6
2	Female	56	LD	16	2.0	2.1
3	Male	60	ED	25	2.4	2.5
4	Male	68	LD	27	2.4	2.4
5	Male	59	LD	24	2.2	2.4
6	Male	60	LD	27	2.1	1.8
7	Male	62	LD	23	1.9	1.9
8	Male	79	ED	20	2.0	2.1
9	Male	59	LD	25	2.3	2.0
10	Male	67	ED	23	2.6	2.4

SCLC, small cell lung cancer; LD, limited disease SCLC; ED, extensive disease SCLC; TMSE, Thai Mental State Examination

Table 2. Dose-volume histogram (DVH) of bilateral hippocampi for each patient

Patient No.	Dose to right hippocampus (Gy)		Dose to left hippocampus (Gy)	
	D2	D100	D2	D100
1	11.6	8.4	11.6	7.8
2	14.5	7.8	14.7	8.0
3	13.3	8.9	13.6	8.9
4	12.7	9.0	12.8	9.1
5	9.9	7.1	9.8	7.6
6	9.9	6.7	9.4	7.1
7	9.8	7.2	10.0	7.3
8	13.5	9.4	13.8	9.0
9	14.1	8.9	12.6	8.6
10	11.1	8.2	10.7	8.0

Table 3. Comparison of TMSE score and hippocampal volumes between baseline and after complete HA-PCI at 3-month and 6-month

Parameters	Baseline	At 3-month	*p*-value	At 6-month	*p*-value
Median TMSE score (IQR)	23 (20-25)	21 (19-26)	0.61	23 (21-27)	0.07
Median Rt hippocampal volume (cc) (IQR)	2.3 (2.0-2.6)	2.3 (1.8-2.7)	0.87	2.2 (2.0-2.7)	0.45
Median Lt hippocampal volume (cc) (IQR)	2.1 (2.0-2.4)	2.2 (1.9-2.6)	0.93	2.2 (2.0-2.5)	0.80

p-value: Wilcoxon Signed Rank test

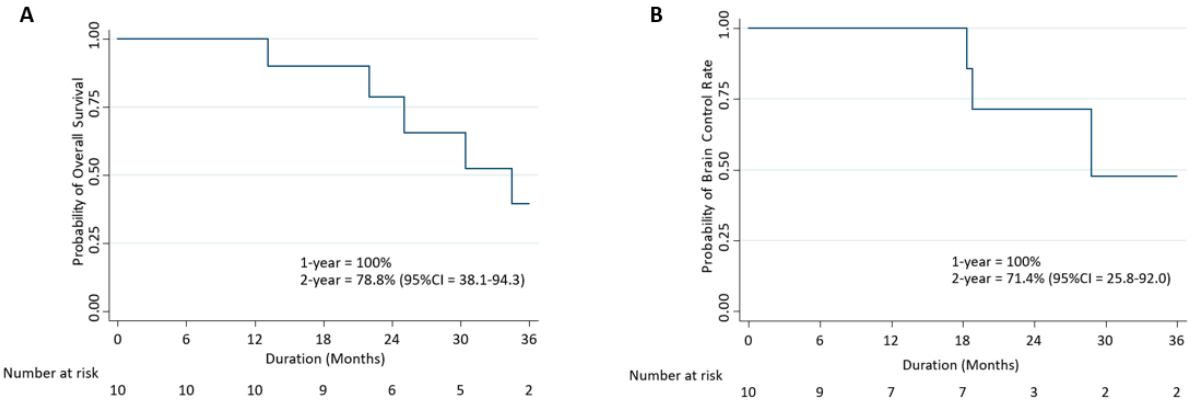

Figure 1. Overall survival (A) and brain control rates (B)

as presented in Figure 1. Brain metastasis was detected from surveillance MRI in patients number 4, 7, and 8 after completing HA-PCI at 20 months, 16 months, and 12 months, respectively. Cerebellum, frontal, and post-central gyrus are the sites of the relapsed brain lesions. All of the patients were asymptomatic and received either whole-brain or salvage stereotactic radiotherapy. All of them also had tumor progression at the lungs and extra-cranial tumor progression, and all died within 6 months after brain lesion recurrence.

DISCUSSION

Small cell lung cancer has a high propensity for intracranial metastasis. In our study, 4 out of 18 patients (22.2%) had brain dissemination detected on MRI screening prior to RT. PCI remains a standard treatment option following chemotherapy, either with or without thoracic RT. A reduced radiation dose to the neural stem cells that reside in the hippocampal region is theorized to protect against neurocognitive impairment from radiation. In this single-center study, there was no statistically significant difference between the

TMSE scores at baseline and after RT completion. Many studies have documented neurocognitive preservation following HA-PCI. In a Spanish national clinical trial, SCLC patients were randomized to undergo either standard PCI or HA-PCI (17). Patients who received HA-PCI experienced less cognitive decline without an increase in the incidence of brain metastases (17). In this study, the incidence of intracranial recurrence was slightly higher than in previous studies. Intracranial recurrence was observed in three out of ten patients in our study. All were located outside the hippocampal region. The landmark NRG Oncology CC003 study conducted in the United States reported an intracranial relapse rate of 14.2% at 12 months (18), while an investigation conducted in South Korea reported a relapse rate of 20.8% (19). However, the rate of intracranial failure in that study did not differ significantly between conventional PCI and HA-PCI groups, and no clinical factor was found to be associated with the intracranial failure (19). In our study, the bilateral hippocampal volumes following HA-PCI did not decrease significantly. A European study found that HA-PCI reduced hippocampal atrophy at 4 and 12 months compared to conventional PCI (20) but no correlation was observed between hippocampal atrophy and memory (20).

The major limitation of this study was the small sample size. We proposed this research in 2018, but participation was inadequate. This may be the result of the coronavirus 2019 (COVID-19) outbreak, which has impeded hospital service by reducing the number of healthcare employees and delaying treatment schedules. In addition, patients and their caregivers were concerned about the spread of these viruses. The small sample size limited the robustness of the data analysis in our study. A previous study with a limited sample size of 20 patients from Johns Hopkins University reported that there was no significant decline in neuropsychological testing results at 6 and 12 months following HA-PCI (21). In that study, the two-year OS was 88%, which is better than our study that found a two-year OS of 78.8%. In addition, an intracranial recurrence was documented in the under-dosed hippocampal avoidance region of 2 patients in this study (21).

CONCLUSIONS

Reduced radiation dose at the hippocampal region during prophylactic cranial irradiation does not increase intracranial relapse in SCLC patients. HA-PCI should be considered as a treatment option which can protect neurocognitive functions without negatively impacting treatment outcome.

ACKNOWLEDGMENTS

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

ADDITIONAL INFORMATION

Author Contribution

B.J.: conceptualization, writing-review and editing this article; W.V.: imaging interpretation; W.N.: radiation dosimetry data collection; P.K.: patient data collection; P.S.: statistical analysis; I.C.: Patient data collection; E.T.: patient data collection; S.C.: patient data collection; W.O.: patient data collection.

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Blood Plasma as a Sampling Model during Drug-induced Thrombocytopenia: Effects of Antioxidants

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ABSTRACT

OBJECTIVE Blood plasma, a complex biological mixture, plays a part in a variety of roles including clotting, defense, and transport, and reflects the overall status of blood components. Drug-induced thrombocytopenia (DIT) is characterized by abnormally low platelet count (below ~150,000 per µL) which can be caused by adverse effects of medications. This study aims to address a basic question, can plasma be employed as a sampling model to assess the oxidative stress (OS) changes and antioxidant status during DIT? The objective was to analyze the antioxidant status and OS in plasma during DIT, and to determine the effects of antioxidant supplementation such as Caripill™, L-Carnitine (LC), and vanillic acid (VA) during DIT.

METHODS Male *Wistar* rats were used as animal models and grouped into control groups (n=5) and thrombocytopenia groups (n=5). Antioxidants were given to the thrombocytopenic and the control rats (50 mg/kg body weight) once a day for 7 days. Blood plasma from both groups was evaluated for total antioxidant capacity, antioxidant enzymes, markers of lipid peroxidation, protein oxidation, and OS.

RESULTS The antioxidants significantly increased the total antioxidant capacity (CUPRAC) and ferric-reducing antioxidant power (FRAP) of the plasma and decreased the levels of conjugate dienes. Caripill™ also reduced lipid peroxidation, significantly elevated protein sulfhydryl and nitrite/nitrate levels, LC elevated lactate dehydrogenase levels, and VA increased superoxide dismutase activity and attenuated lipid peroxidation during DIT.

CONCLUSIONS The antioxidants Caripill™, vanillic acid, and L-carnitine were demonstrated to be beneficial during DIT and to have prospects in alternate therapeutics. This study confirms that plasma can be utilized as a sampling model to study changes during OS situations.

KEYWORDS antioxidants, caripill, L-carnitine, oxidative stress, plasma, thrombocytopenia, vanillic acid

INTRODUCTION

Oxidative stress (OS) during thrombocytopenia is one of the most important factors that can affect platelet count. Plasma is representative of the entire OS microenvironment of the whole blood. Proteins are the most abundant substances in plasma and play a part in a variety of roles including clotting, defense, and transport (1, 2). An imbalance of reactive species and endogenous antioxidants in the plasma causes damage to

biological molecules during disease conditions (3, 4). However, endogenous antioxidants can neutralize free radical intermediates and inhibit their oxidation to a certain extent (5). Antioxidant supplementation has gained acceptance as an alternative to complement the current therapies. Alternative therapeutics can be an effective tool for treating secondary complications and can be integrated with conventional medicine.

Drug-induced thrombocytopenia (DIT) is a condition induced by therapeutic antibiotics, cardiovascular drugs, and anti-cancer agents (6, 7). These agents can affect the platelet count by their cytotoxic effects on bone marrow megakaryocytes, thereby reducing platelet synthesis, or by accelerating the clearance of circulating platelets (8). Some of the important drugs involved in inducing thrombocytopenia are balhimycin, vancomycin, sulfonamides, trifluoperazine, carmustin, phenacetin, cisplatin, penicillin, methotrexate, lovastatin, cyclophosphamide, quinine, quinidine, methicillin, and hydroxyurea (9).

Herbal supplements have proven to be beneficial in treating thrombocytopenic conditions. For example, *Carica papaya* leaf extract contains flavonoids and polyphenols and is an active pharmaceutical ingredient of Caripill™ tablets (10). It aids the increase in platelet count in dengue infection and other thrombocytopenic conditions (11, 12). Vanillic acid (VA), a naturally occurring phenolic compound found in many edible plants and fruits possesses free radical scavenging, anticancer, antimutagenic, and antiangiogenic effects with multiple pharmacologic functions such as antimicrobial, anti-filarial, anti-inflammatory, and antioxidant activities. It can directly scavenge hydroxyl radicals and other cellular oxygen species due to the presence of the carboxyl group (13). It has proven to be beneficial in ameliorating oxidative stress by reducing lipid peroxidation products and significantly restoring enzymatic and non-enzymatic antioxidant systems (14, 15). L-carnitine (LC) is a non-protein amino acid and a free radical scavenger. The carbonyl group stabilizes free radicals on α-carbon. It prevents oxidative damage to antioxidant enzymes by chelating metal ferrous ions, thereby interfering with reactive species formation (16). The effect of LC during oxidative stress has been well demonstrated in different biological systems (17, 18).

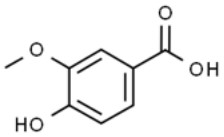
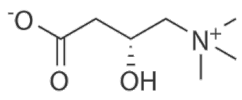
This study aims to address a basic question: can plasma be employed as a sampling model to assess the OS changes and antioxidant status? This study was designed to (i) analyze the antioxidant status in plasma under thrombocytopenic conditions, (ii) determine the status of protein oxidation and lipid peroxidation in plasma under thrombocytopenic conditions, and (iii) assess the effects of antioxidants such as Caripill™, L-carnitine and vanillic acid as supplements during thrombocytopenia. The structures and composition of the studied antioxidants are listed in Table 1.

METHODS

Chemicals

Caripill™ (*Carica papaya* leaf extract tablets, Micro Lab Limited, Bangalore, India) and hydroxyurea (Hydrea, Sarabhai Chemicals, Gujarat, India) were purchased from a local pharmacy. 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA), dinitrophenyl hydrazine (DNPH), epinephrine, bathocuproinedisulfonic acid disodium salt (BCS) and Griess reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA); 2,4,6-Tripyridyl-s-triazine (TPTZ), L-carnitine (LC) and vanillic acid (VA) were purchased from HiMedia Laboratories (Maharashtra, India). All other

Table 1. Antioxidants supplemented during drug-induced thrombocytopenia

Antioxidant	Composition/structure
Caripill™ (10)	Flavonoids Phenolic compounds Papain Chymopapain Cystatin Tocopherol Ascorbic acid Cyanogenic-glucosides Glucosinolates Cardiac glycosides Anthraquinones Carpaine Pseudocarpaine
Vanillic acid (13)	
L-carnitine (16)	

chemicals were reagent grade and organic solvents were spectral grade.

Instruments

All absorbance measurements were carried out using a PC-based Double Beam Spectrophotometer 2202 (Systronics, Gujarat, India) with 10 mm quartz cuvettes. All 96-well microplates were read using a LISA Plus microplate ELISA reader (Aspen Diagnostics, Delhi, India). A refrigerated centrifuge (REMI, Mumbai, India) was used for centrifugation.

Animal care

Wistar rats were used in this study due to the similarity in their physiology with that of humans and to avoid baseline variations as the rats are bred under controlled conditions. Ethics approval for animal studies was obtained from the Institutional Animal Ethics Committee, Nargund College of Pharmacy, Bangalore, India (Ethics approval number: IAEC/NCP/93/2015).

Four-month-old male *Wistar* rats (~200-240 g) were acclimatized for a week in animal house conditions prior to the study. The rats were housed in cages (five in each cage) at 28 ± 1 °C with a 12/12 h light and dark cycle. They were fed lab chow and tap water ad libitum.

Experimental design

Hydroxyurea was orally administered to each rat at 400 mg/kg body weight (thrombocytopenia group) once daily for 10 days (11). The antioxidants (50 mg/kg body weight) were orally administered to the respective groups once daily for 7 days.

The animals were divided into the following groups (n=5): group i – controls; group II - control + L–carnitine (C+LC); Group III - Control + Caripill™ (C+CP); group IV - control + vanillic acid (C+VA); group V - thrombocytopenia (Thr); group VI - thrombocytopenia + L–carnitine (Thr+LC); group VII - thrombocytopenia + Caripill™ (Thr+CP); and group VIII - thrombocytopenia + vanillic acid (Thr+VA).

Sampling of blood

The *Wistar* rats were anesthetized with ether and then restrained in dorsal recumbency. A syringe needle was inserted into the heart, slightly below the xiphoid cartilage and to the left of the midline. Blood was drawn into collection tubes containing citrate phosphate dextrose adenine-1 (CPDA-1) as an anti-coagulant (19).

Isolation of plasma

Whole blood was centrifuged at room temperature (RT) for 20 min at 2,000 rpm to separate the platelet-rich plasma (PRP). The PRP was then centrifuged at 4,000 rpm at 22 °C for 15 min. The resulting supernatant containing platelet-poor-plasma was stored for further analysis at -20 °C (20).

Superoxide dismutase (SOD)

SOD activity in the plasma samples was analyzed according to the method of Misra and Fridovich, 1972 (21). Plasma samples were added to carbonate buffer (0.05 M, pH 10.2, containing 0.1 mM EDTA). Epinephrine was added to this mixture and absorbance was measured immediately at 480 nm. SOD activity was determined as the amount of enzyme that can inhibit the oxidation of epinephrine by half, which is equal to 1 unit.

Catalase (CAT)

Catalase activity was measured according to Aebi, 1984 (22). Absolute ethanol (10 µL) was added to the plasma samples then the mixture was incubated on ice for 30 min. To this, H_2O_2 (66 mM) in phosphate buffer (0.1 M) was then added and the absorbance was measured immediately at 240 nm. Catalase activity was measured using the molar extinction coefficient 43.6 Mcm^{-1}.

Cupric-ion reducing antioxidant capacity (CUPRAC)

The plasma samples were treated with 0.25 mM BCS (dissolved in 10 mM phosphate buffer solutions) and the absorbance was spectrophotometrically measured at 490 nm. 0.5 mM $CuSO_4$ was added to the samples and the mixture was incubated at room temperature for 3 min. The reaction was terminated by adding EDTA (0.01 M). Absorbance was measured at 490 nm using uric acid in 1 M NaOH as the standard (23).

Thiobarbituric acid reactive substances (TBARS) assay

TBARS level in plasma was measured according to the method of Olas et al., 2006 (24). Plasma

samples were cooled on ice for 10 min and then 20% (v/v) cold trichloro acetic acid (TCA) in 0.6 M HCl was added. The mixture was centrifuged for 15 min, then 0.12 M thiobarbituric acid (TBA) was added to the supernatant. This was boiled for 20 min, after which absorbance was recorded at 532 nm using a microplate reader.

Conjugate dienes (CD)

Plasma was treated with diethyl ether/ethanol mixture (1:3 (v/v)) and the mixture was vortexed. It was centrifuged at 4,000 rpm. The absorbance of the supernatant was read at 235 nm (25).

Protein carbonyls (PrC)

PrC in plasma were analyzed according to Reznick & Packer, 1994 (26). Plasma samples were incubated with 10 mM dinitrophenyl hydrazine (DNPH) in the dark for 1 h at room temperature. TCA (20%) was added to the samples which were then incubated on ice for 10 min. The mixture was centrifuged at 3,000 rpm. The pellets were washed with ethanol (ethyl acetate) [1:1 (v/v)] until the color disappeared. The final precipitate was dissolved in 6 M guanidine HCl. Absorbance was measured at 370 nm. Protein carbonyl content was calculated using the absorption coefficient 22,000 $M^{-1}cm^{-1}$.

Protein sulfhydryls (SH)

Plasma samples were mixed with 0.08 mol/L sodium phosphate buffer (pH 8.0, with 0.5 mg/mL Na_2-EDTA and 2% sodium dodecyl sulfate) and the mixture was vortexed. DTNB was added to the mixture which was incubated for 15 min at room temperature. Absorbance was measured at 400 nm. SH in the plasma samples was calculated using a molar absorptivity of 13,600 $M^{-1}cm^{-1}$ (27).

Ferric reducing ability of plasma (FRAP)

FRAP assay was performed using the method of Benzie and Strain, 1996 (28). Plasma samples were added to a freshly prepared FRAP reagent (300 mM acetate buffer [pH 3.6], 10 mM tripyridyltriazine, and 20 mM $FeCl_3$). The reaction mixture was incubated for 5 min at 37 °C and absorbance was read at 593 nm. FRAP was determined using the extinction coefficient 21,250 mM^{-1} cm^{-1}.

Nitrites and nitrates

Plasma samples were mixed with Griess reagent (100 µL) and incubated for 30 minutes in the dark at room temperature. Absorbance was read at 520 nm. The amount of nitrites present in the plasma sample was determined using sodium nitrite as the standard (29).

Nitrate levels were determined following Y/egin et al., 2015. Plasma samples were treated with $CuSO_4$ (50 µg/mL), $(NH_2)_2SO_4$ (1 mg/mL), NaOH (8 mg/mL), and Griess reagent. This mixture was vigorously shaken and the absorbance was measured at 520 nm (30). The results are expressed as a (nitrites)/(nitrates) ratio.

Lactate dehydrogenase (LDH)

Plasma samples were treated with a mixture of reagent 1 (containing 80 mM Tris, 200 mM NaOH, and 1.6 mM pyruvate) and reagent 2 (0.2 mM nicotinamide adenine dinucleotide). This mixture was incubated at 37 °C for 5 min. The decrease in absorbance was measured at 340 nm for 3 minutes (31).

Protein estimation

The protein content in the plasma samples was estimated according to Lowry et al., 1951 (32). The diluted plasma sample (20X) was mixed with alkaline copper sulfate (50:1 mixtures of A: 2% Na_2CO_3 in 0.1 N NaOH and B: 0.5% $CuSO_4$ in 0.1 N sodium potassium tartrate). The mixture was incubated for 15 min at room temperature. Folin's reagent was added and the mixture was incubated for 30 min at room temperature. The optical density was measured colorimetrically at 660 nm.

Statistical analyses

Results are expressed as mean ± SEM (n=5). One-way ANOVA analysis between the groups for all the parameters was performed based on the Bonferroni post-hoc test using GraphPad Prism 6 software and $p < 0.05$ was considered statistically significant.

RESULTS

Platelet counts were 8.88 ± 0.24 lakhs/mm^3 (control group) and 1.86 ± 0.3 lakhs/mm^3 (Thr group). Platelet count increased by 5-fold in the Thr+CP and Thr+VA groups, and 3-fold in the Thr+LC group compared to the Thr group.

Superoxide dismutase (SOD)

SOD levels in plasma were maintained across all control groups. The Thr+VA group showed a significant increment of 143% compared to the C+VA group ($p < 0.05$). SOD increased 92% and 134% in the Thr+VA group with controls and the Thr group, respectively (Figure 1A). The control group was set as 100%.

Catalase (CAT)

Variations in CAT were similar in the control and experimental groups (Figure 1B).

Cupric ion reducing antioxidant capacity (CUPRAC)

The changes observed in CUPRAC were similar in the control and experimental groups (Figure 2A).

Ferric reducing ability of plasma (FRAP)

Changes in FRAP were statistically significant between the groups. FRAP increased significantly by 74% in C+CP and 81% in C+VA groups against controls ($p < 0.05$). FRAP increased significantly by 60-80% in the Thr+LC, Thr+CP, and Thr+VA groups compared to controls ($p < 0.05$). There were increments of 20% and 50% in the C+LC and Thr groups with respect to controls, respectively (Figure 2B).

Lipid peroxidation (LPO) products

Variations in TBARS levels were observed among the groups, but were similar (Figure 3A). Changes between the groups in conjugated dienes were statistically significant. There was a significant decrement of approximately 30% in all experimental groups with respect to the controls ($p < 0.05$) (Figure 3B).

Protein oxidation products

Protein carbonyls (PrC) showed statistically significant changes in the different control groups. All the control groups supplemented with antioxidants (C+LC, C+CP, and C+VA) showed a significant reduction of ~40% compared to controls ($p < 0.05$). PrC also increased in the Thr+VA group by 210% with respect to the C+VA group (Figure 4A). Protein sulfhydryl (SH) was significantly higher (70%) in the Thr+CP group with respect to the controls ($p < 0.05$). There were increments of 35%, 45%, and 60% in the Thr, Thr+LC and Thr+VA groups, respectively, compared to controls. SH levels were also higher by 20%, 40%, and 65% in C+LC, C+CP and C+VA groups, respectively, compared to controls (Figure 4B).

Nitrite/nitrate ratio

The control group was set as 100%. The nitrite/nitrate levels were similar in plasma; however, there was a statistically significant increase in the Thr+CP group (140%) compared to controls. A 70% increment was observed in the Thr+CP group with respect to the Thr group, and the ratio increased by 80% in the Thr+CP compared to the C+CP group (Figure 5).

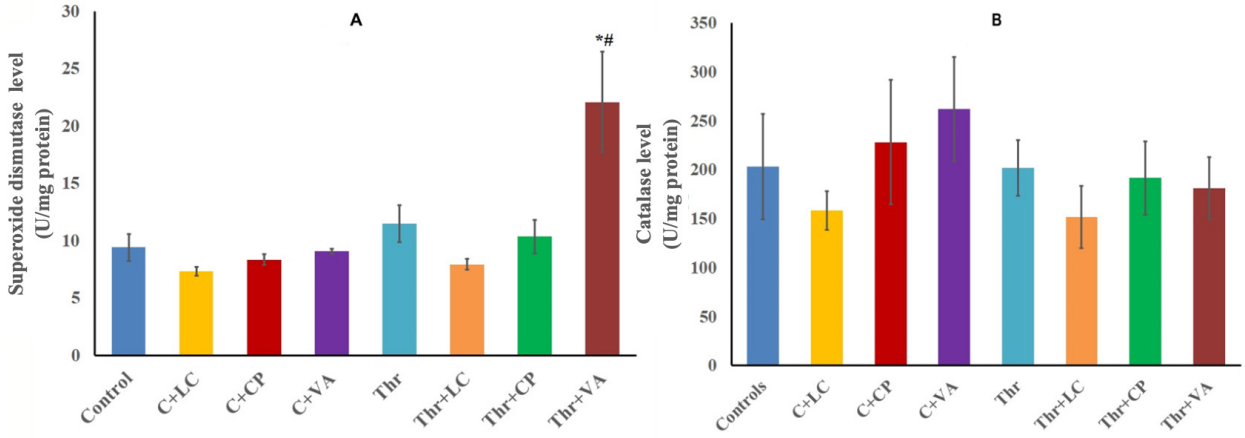


Figure 1. Superoxide dismutase (A) and catalase (B) in plasma of rats with induced thrombocytopenia
C+LC- Control + L-carnitine; C+CP- Control + Caripill™ ; C+VA- Control + vanillic acid; Thr- thrombocytopenia; Thr+LC- thrombocytopenia + L-carnitine; Thr+CP-thrombocytopenia + Caripill™ ; Thr+VA- thrombocytopenia + vanillic acid
'*' represents groups with statistically significant differences ($p < 0.05$) compared to the controls.
'#' represents groups with statistically significant differences ($p < 0.05$) compared to the thrombocytopenia.

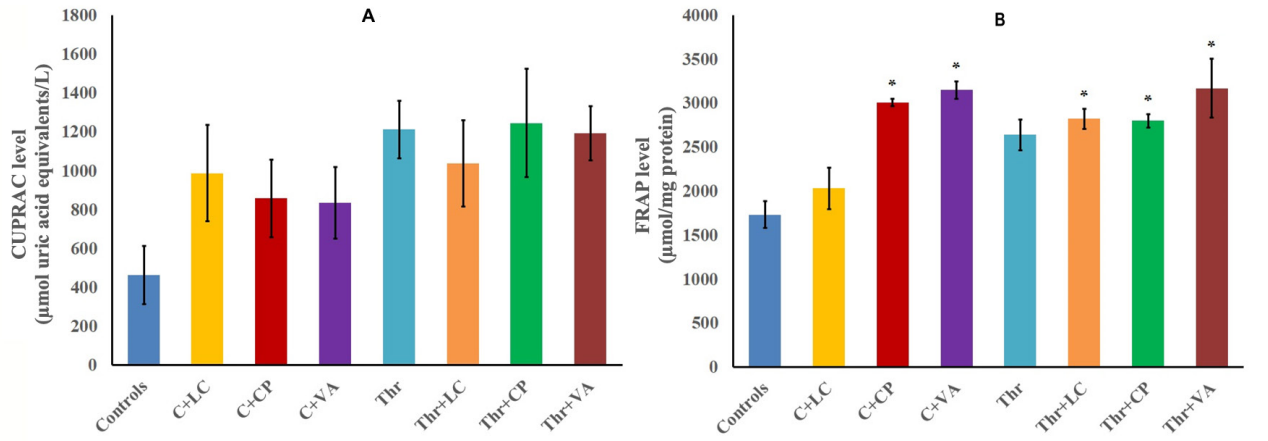


Figure 2. Cupric ion reducing antioxidant capacity (CUPRAC) (A) and ferric reducing ability of plasma (FRAP) (B) in plasma of rats with induced thrombocytopenia
C+LC- Control + L-carnitine; C+CP- Control + Caripill™ ; C+VA- Control + vanillic acid; Thr- thrombocytopenia; Thr+LC- thrombocytopenia + L-carnitine; Thr+CP- thrombocytopenia + Caripill™; Thr+VA- thrombocytopenia + vanillic acid
'*' represents groups with statistically significant differences ($p < 0.05$) compared to the controls.

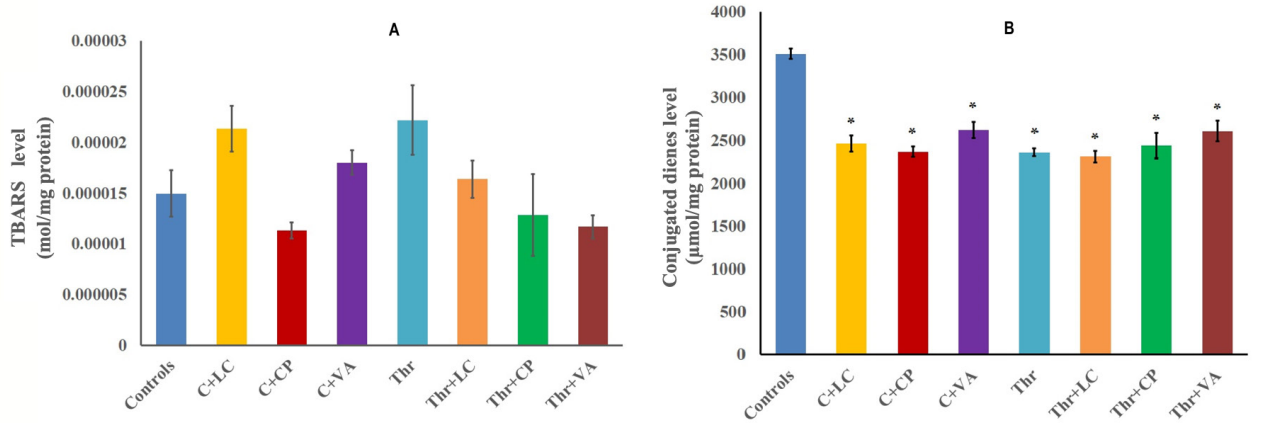


Figure 3. TBARS (A) and Conjugated Dienes (B) in plasma of rats with induced thrombocytopenia
C+LC- Control + L-carnitine; C+CP- Control + Caripill™ ; C+VA- Control + vanillic acid; Thr- Thrombocytopenia; Thr+LC- Thrombocytopenia + L-carnitine; Thr+CP-Thrombocytopenia + Caripill™; Thr+VA- Thrombocytopenia + vanillic acid
'*' represents groups with statistically significant differences ($p < 0.05$) compared to the controls

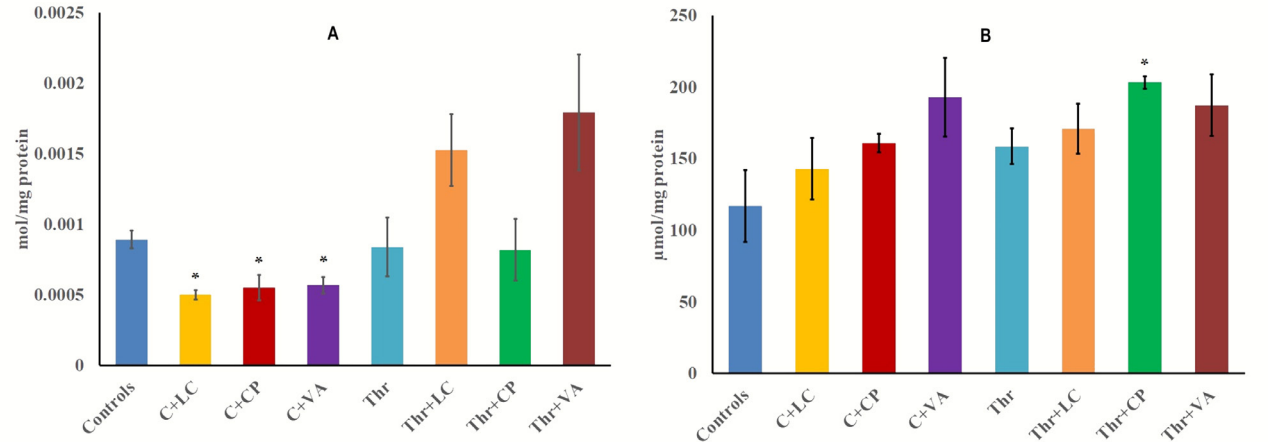


Figure 4. Protein carbonyls (PrC) (A) and protein sulfhydryl (P-SH) (B) in plasma of rats with induced thrombocytopenia
C+LC- Control + L-carnitine; C+CP- Control + Caripill™ ; C+VA- Control + vanillic acid; Thr- Thrombocytopenia; Thr+LC- Thrombocytopenia + L-carnitine; Thr+CP-Thrombocytopenia + Caripill™; Thr+VA- Thrombocytopenia + vanillic acid
'*' represents groups with statistically significant differences ($p < 0.05$) compared to the controls.

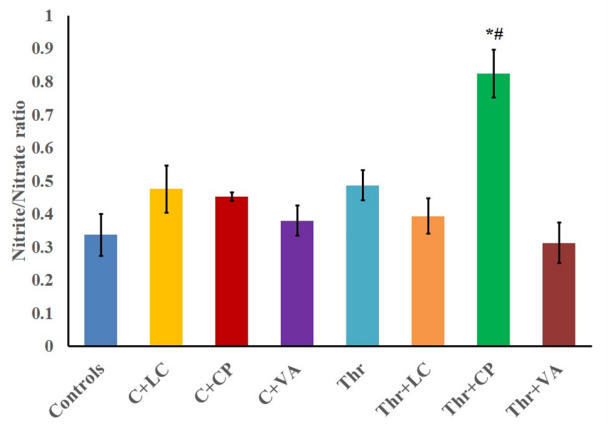


Figure 5. Nitrites/nitrates ratio in plasma of rats with induced thrombocytopenia
C+LC- Control + L-carnitine; C+CP- Control + Caripill™; C+VA- Control + vanillic acid; Thr- Thrombocytopenia; Thr+LC- Thrombocytopenia + L-carnitine; Thr+CP- Thrombocytopenia + Caripill™ ; Thr+VA- Thrombocytopenia + vanillic acid
'*' represents groups with significant differences ($p < 0.05$) compared to the controls.
'#' represents groups with statistically significant differences ($p < 0.05$) compared to the thrombocytopenia.

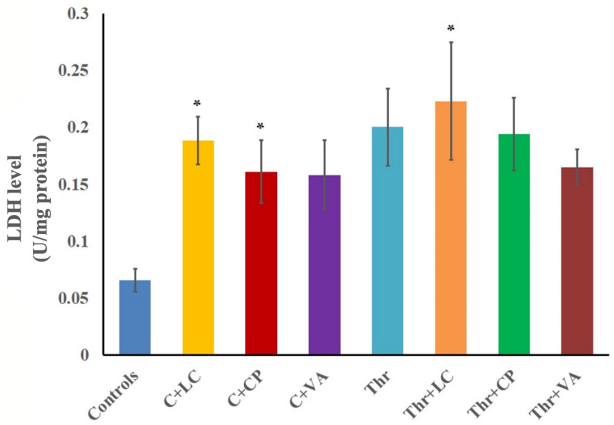


Figure 6. Lactate dehydrogenase (LDH) in plasma of rats with induced thrombocytopenia
C+LC- Control + L-carnitine; C+CP- Control + Caripill™; C+VA- Control + vanillic acid; Thr- thrombocytopenia; Thr+LC- thrombocytopenia + L-carnitine; Thr+CP- thrombocytopenia + Caripill™ ; Thr+VA- thrombocytopenia + vanillic acid
'*' represents groups with statistically significant differences ($p < 0.05$) compared to the controls

Lactate dehydrogenase (LDH)

The control group was set as 100% and the changes in LDH in the other groups were statistically significant. Groups supplemented with L-carnitine (C+LC) and Caripill™ (C+CP) antioxidants increased the LDH level significantly by 190% and 150%, respectively, compared to controls ($p < 0.05$). LDH level also significantly increased by 240% in the Thr+LC group compared to the controls ($p < 0.05$). There were increments of 140%, 200%, and 150% in the C+VA, Thr+CP and Thr+VA groups compared to controls, respectively (Figure 6).

DISCUSSION

Endogenous antioxidant machinery, comprised mainly of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), forms the first line of defense against reactive oxygen species (ROS) (33). SOD is an enzyme that acts on superoxides and converts them into less toxic H_2O_2. SOD levels were elevated in Thr+VA, indicating that VA supplementation in thrombocytopenic rats activated SOD and thereby scavenged superoxides (34). Catalase terminates the reaction started by SOD by degrading H_2O_2 to H_2O and O_2. H_2O_2 can also be scavenged by GPx. GPx and CAT have the same action upon H_2O_2, but GPx is more efficient during periods of low ROS levels, whereas CAT acts more effectively during high H_2O_2 concentrations. Similar variations in CAT during thrombocytopenia indicate that GPx can act on lower levels of H_2O_2 as GPx is the first line of defense against H_2O_2 (35, 36).

The antioxidant potential of the plasma was assessed based on CUPRAC and FRAP assays. CUPRAC assay provides the concentration of non-enzymatic antioxidants in the plasma sample. Similar levels indicated that endogenous non-enzymatic antioxidant defenses had maintained antioxidant potential in the plasma (37). The molar concentration of antioxidants present in plasma denoted by FRAP signifies that both the antioxidants and endogenous antioxidant systems acted to provide better protection against oxidative stress (38). Elevated FRAP in C+CP, C+VA, Thr+LC, Thr+CP and Thr+VA suggest that all three antioxidants (LC, Caripill™, and VA) show multiple mechanisms of scavenging free radicals, chelation of metal ions, and inhibition of free radicals and lipid hydroperoxides formation (39). Nonetheless, VA was the most effective antioxidant for improving the overall antioxidant capacity in plasma during thrombocytopenia. It has a carboxyl group that can transfer electrons or donate protons, and can therefore directly scavenge hydroxyl radicals and other cellular oxygen species (13). LC chelates metal ferrous ions and inhibits ROS formation. Its

carbonyl group stabilizes free radicals on α-carbon and protects cells against oxidative damage from ROS and RNS (16).

Oxidative stress in a biological system can be measured by assessing products of lipid peroxidation such as TBARS and conjugated dienes, and protein oxidation products such as sulfhydryls and carbonyls (4). Lipid peroxidation levels can be used to assess the damage caused to cell membranes such as membrane fluidity alteration, permeability, and integrity (40). Lipids upon oxidation give rise to products such as conjugate dienes and TBARS. CD is used as a marker for the identification of non-specific lipid peroxidation caused by free radicals. TBARS measure malondialdehyde (MDA) present in the plasma, one of the low molecular weight end products generated due to the decomposition of some primary and secondary lipid peroxidation products (41). Conjugated dienes levels were decreased in the experimental groups compared to the controls. Changes in TBARS levels were similar in all the experimental groups. This indicates that lipid peroxidation was negligible due to the free radical scavenging activity of antioxidants and the antioxidant capacity of the plasma. This was also corroborated in our results by CUPRAC levels. Caripill™ was the most effective in protecting the plasma from lipid peroxidation in both the C+CP and Thr+CP groups. This can be attributed to the antioxidant activity of the flavonoids and other phenolic compounds present in its active pharmaceutical ingredient, *C. papaya* leaf extract (10).

Carbonyl groups can be formed by different mechanisms, one of them being the oxidative cleavage of protein backbone, particularly at glutamyl side chains, and also oxidative deamination of lysine. The attack of hydroxyl radicals on proline, arginine, lysine and threonine side chains also leads to the formation of carbonyl groups (42). The generation of carbonyls in all control groups supplemented with antioxidants decreased, suggesting that antioxidants help protect protein from oxidation whereas similar protein carbonyl levels were maintained in the Thr groups during the disease condition, indicating that antioxidant supplementation can protect plasma against protein carbonyl formation. Protein sulfhydryl levels contribute significantly to the antioxidant status of plasma (43). Sulfhydryl (SH) becomes oxidized to disulfide, which can be reversed by the action of ROS. ROS are mainly present in the cysteine components of proteins during low glutathione concentrations (44). SH increased in thrombocytopenic rats treated with Caripill™ compared to controls, indicating reduced oxidative stress. Caripill™ protected the SH from oxidation more effectively than both LC and VA, suggesting that CP is the most potent in maintaining SH in a reduced state.

The direct measure of reactive nitrogen species such as nitric oxide radicals is tedious due to their radical nature and short half-life. These radicals are generated in plasma by specific nitric oxide synthases (NOSs). For that reason, measurement of the stable end products of NO radicals, such as nitrite, and nitrate, in plasma is most often used for estimating NO radicals (45). One of the main factors interfering with NO is superoxide anions, which favor the generation of peroxynitrite anions. NO and its metabolites stimulate and inhibit lipid peroxidation reactions (46). An increase in the nitrite/nitrate ratio of Thr+CP showed that more RNS were generated in this group. Lactate dehydrogenase is considered one of the indicators of tissue/cell damage (47). Similar changes in the Thr+VA and C+VA groups suggest that VA supplementation is most efficient in maintaining LDH levels in thrombocytopenic conditions similar to the controls.

CONCLUSIONS

Plasma has been demonstrated to be an ideal sample as plasma is of biological origin, it is more appropriate to use sample instead of material for studying oxidative stress during DIT as it represents all the changes occurring in the blood during DIT. This study confirms that Caripill™ is effective in improving overall antioxidant status (FRAP) in plasma, reducing lipid peroxidation, and increasing the protein sulfhydryls in thrombocytopenia groups. VA upregulated SOD and attenuated lipid peroxidation more effectively in thrombocytopenia groups compared to non-thrombocytopenic controls. Nonetheless, all the antioxidants enhanced the antioxidant status of plasma in both control and thrombocytopenic conditions as seen in the CUPRAC and FRAP results. The antioxidants Caripill™, VA, and LC have been shown to be beneficial during DIT. Antioxidant

supplementation can be beneficial in thrombocytopenic conditions which opens new avenues for employing antioxidants in alternate therapeutics.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

ADDITIONAL INFORMATION

Author contribution

V.R.: conceptualization, Visualization, methodology, validation, writing–review and editing; A.B.A: writing–original draft preparation; A.H., F.A., O.O.L, P.D., S.S.R.: investigation, data curation, writing- original draft preparation; MM: supervision, investigation.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Estimation of Post-mortem Interval Based on Livor Mortis using a Colorimeter in Thai Populations

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ABSTRACT

OBJECTIVE Livor mortis is a helpful and widely used method of estimating postmortem interval (PMI) in Thailand. This study aimed to investigate the value of a colorimeter as a tool for estimating the PMI.

METHODS The color of livor mortis and control skin in 80 cadavers whose PMI was within 12 hours was measured by a colorimeter. The L* (brightness), a*b* (chroma and hue), and ΔE* values were compared to the control skin values. Statistical analysis was performed to determine the relationship between PMI and skin color before and after application of a specific pressure.

RESULTS The results showed that colorimetric parameters were only weakly correlated with the PMI. An univariable analysis of ΔE* values was performed and showed good discriminatory power, with an area under the ROC curve of 0.82. The recommended cut-off value of ΔE* was 14 for the discrimination between early PMI (less than 6 hours) and late PMI (6-12 hours), in which the sensitivity and specificity were 72.5% and 80%.

CONCLUSIONS The findings in this study reinforce the utility of colorimetric measurements in PMI estimation. With additional study and a larger sample size, the estimation of PMI could be established for general use in forensic practice.

KEYWORDS livor mortis, colorimeter, time since death, taphonomy, forensic pathology

INTRODUCTION

In the field of forensic science, the post-mortem interval (PMI) refers to the period between the occurrence of death and the discovery of a body. The accurate determination of the PMI is of the utmost priority, as it facilitates the identification of human remains and contributes to an extensive examination of potential causes of death. Furthermore, the establishment of the PMI assumes significance in discerning potential criminal acts and facilitating the determination of appropriate legal consequences (1-3). The estimation of the PMI relies on the examination of various post-mortem changes exhibited by a cadaver. These alterations include physical changes (body cooling and livor mortis), physicochemical processes (cadaveric stiffening), metabolic reactions (supra-vital reactions), decomposition, and the influence of insect activity (4-6).

Livor mortis is a dark-purple staining of the skin resulting from the gravitational accumulation of blood within the vascular system of the body's dependent regions (7-9). After circulatory arrest, livor mortis develops as one of the early postmortem alterations. Within one hour after death, livor mortis becomes visible as pink patches that result

in a progressive and consistent confluence with an increasing PMI, usually reaching its maximum coloration and becoming fixed at variable times after death (8). For that reason, livor mortis is a useful and popular method for estimating PMI (9), and it is a common practice in Thailand. To assess livor mortis, forensic pathologists frequently employ visual observation of its color and distribution (10). Nevertheless, it is challenging to give a precise and accurate description of the color and level of hypostasis due to the high subjectivity of visual color recognition and estimation of the progression of livor mortis. To measure the color of livor mortis more accurately, a number of objective techniques have been attempted (11-18). One technique is colorimetric analysis which is employed as an adjunctive tool to facilitate the estimation of the PMI.

Previous studies have demonstrated the utilization of a tristimulus colorimeter for assessing skin color changes caused by livor mortis (11, 12, 15, 18). Vanezis found that there was a linear relationship between the fading color of hypostasis and the time during which the measurements were carried out (11). Kaatsch and Nietert used a colorimeter to measure pressure-induced color changes in livor mortis. They highlighted the potential usefulness of colorimetric measurement in estimating the PMI based on initial measurements on cadavers, describing the regular course of color changes in livor mortis with the application of increasing pressure (12). Vanezis and Trujillo also emphasized that livor mortis is particularly useful for estimating the PMI within the first 48 hours, as the rate of color change is more pronounced during this period. After that time, the rate of change becomes reduced or non-existent, and by 72 hours, livor mortis has typically become fixed in the majority of cases (15).

Although numerous novel methods have been created which potentially provide more accurate PMI estimation and that can be applied universally (19-21), none have been designed for specific geographic regions (22). Climatic and environmental settings can influence the rate and pattern of postmortem change (22, 23). Therefore, region-specific studies should be conducted. Unfortunately, no taphonomic research focusing on the relationship between the rate of change of livor mortis and PMI has been conducted in tropical zones such as Thailand. In addition, variations in skin color exist among different ancestral populations, potentially reducing the accuracy of colorimetric measurement of livor mortis in Thai and other populations.

This study aimed to investigate the usefulness of the colorimetric measurement of livor mortis in accurately determining the time since death in Thailand, thereby giving further information to help forensic pathologists determine the PMI of bodies found in tropical countries.

METHODS

The study sample

This study site was a metropolitan area of Bangkok, the capital city of Thailand. The region experiences a tropical climate, with an average daily temperature of 28.0 °C. It is classified as Aw according to the Köppen-Geiger classification system (24). This classification means Bangkok is an equatorial savanna with a dry winter. At the time of the measurements, the average environmental temperature ranged from 26 °C to 31 °C and humidity ranged from a minimum of 74% to a maximum of 85%.

This prospective study was conducted on cadavers examined in the Department of Forensic Medicine, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand. From October 2022 to September 2023, 80 forensic autopsy cases were selected for this study. An informed consent form was obtained from legal heirs before data collection. Cadavers with the following features were excluded from this study: 1) an indeterminate time of death, 2) signs of decomposition, 3) a history of anemia or bleeding tendency, 4) not lying in a supine position, and 5) a history of significant hemorrhage from trauma or disease. Pertinent demographic information including sex, age, underlying disease, time and date of death, location of livor mortis, and posture of the cadaver were recorded. The causes of death were determined following complete autopsy examinations and extensive police investigations. The PMI data was checked with hospital records and police investigative records to help ensure the accuracy and consistency of the information.

The PMI was known for all cases and ranged between 1 hour and 12 hours. According to a previous pilot study in Thailand, the level of lightness

of postmortem livor mortis becomes fixed 12 hours after death.

Colorimetric measurement

Skin color measurements were conducted using a portable colorimeter (FRU® WR-18). The instrument was set up including a standard D65 light source, illumination mode 8/d, light mode SCI, observer = Commission Internationale de l'Eclairage (CIE) 10°, color space CIE1976 LAB, and the 8 mm diameter caliber. The instrument was calibrated using a black-and-white calibrator prior to each measurement.

In some cases, a cadaver had been left in an environment with an unknown temperature between the time of death and measurement. At the autopsy room, measurements were carried out at a temperature of 25 °C to 30 °C. Cadavers were turned from a supine position to one side, and the left and right scapulae at the areas inferior to the lateral aspect of the scapular spine were measured three times (Figure 1). These regions were selected due to their higher probability of onset of livor mortis and ease of application of the colorimeter (18). Colorimetric data were measured with minimal force at a right angle to the area of the livor mortis. This initial set of data from the area before the application of pressure was called the 'before-pressure group' or 'BP' group.

Following that, pressure was applied on the same location of livor mortis by pressing the measuring head of the dynamometer (Force Meter SF-100) at a force of 1.5 kg/cm^2 for 3 seconds. These parameters are the most appropriate values for inducing blanching in livor mortis (18). Colorimetric measurements were then taken on the pressed area. This new data set was referred to as the 'after-pressure group' (or 'AP' group). The control colorimetric data were determined by measuring normal skin at the anterior chest (Figure 1). The average data of three scans was used to create each control. The end result was 3 sets of data (control, BP, and AP) for each cadaver which were then analyzed in this study.

Color analysis

The CIE L*a*b* system was used to quantitatively evaluate the color of livor mortis in a three-dimensional space. The L* value denotes brightness with a range of 0-100, where 0 is black and 100 is white. The proportions of different colors are represented by the chroma coordinates a* and b*. A positive value of a* denotes a red component, while a negative value denotes a greenish one. A yellow color is represented by a positive value of b*, whereas a blue color is represented by a negative value. The individual typology angle determination (ITA) was caluclated using the formula: °ITA = [arctan(L*-50)/b*] × 180/3.14159. This allows classificaton skin color types into six groups, from very light to dark skin color: very light > 55°, light 55° to > 41°, intermediate 41° to > 28°, tan 28° to > 10°, brown 10° to > -30° and dark ≤ -30° (25).

In order to evaluate the colorimetry of livor mortis in all three values, L*, a*, and b* were observed before and after the application of pressure and were compared to the colorimetry of the control region. The ΔL*, Δa*, and Δb* colorimetric values were obtained by subtracting the livor mortis values from the control value. In addition, the color difference value (ΔE*) was used to encompass all color differences in a single arithmetic value. The equation used is ΔE* = [(ΔL*)2 + (Δa*)2 + (Δb*)2]1/2. The ΔE* value range is 0 to 100, with 0 indicating very little color change and 100 indicating extreme distortion. The lower the ΔE*, the harder it is to distinguish colors. The greater ΔE*, the more dissimilar two colors are (26).

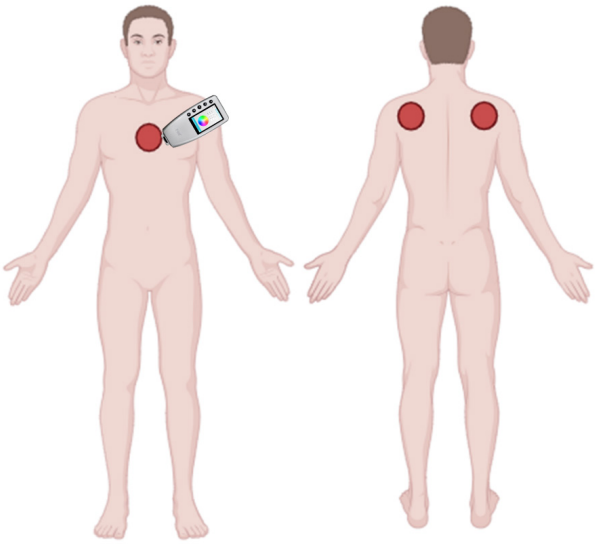

Figure 1. Anatomical landmarks on cadavers from which color measurements were obtained (red circles). This image was created with BioRender (biorender.com)

Statistical analysis

The sample data consisted of descriptive statistics, with continuous data shown as mean, standard deviation (SD), and minimum-maximum values (range). Categorical information is displayed as a number and a percentage. Normal probability plots were used to verify the normality of the colorimetric data. To investigate the relationship between measured color value and PMI, simple correlation was calculated using the Pearson correlation coefficient. Logistic regression analysis was used to analyze the association of PMI and colorimetric parameters as ROC curves and the area under the curves. A cutoff score of the most statistically significant colorimetric parameter was selected based on the sensitivity and specificity of the ROC curve.

All statistical analyses were carried out using SPSS version 25 software (IBM, USA). Statistical significance was defined as a p-value of less than 0.05.

Ethical approval was obtained from the Ethics Committee of the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand (SIRB Protocol No.652/2565 (IRB1), 8 December 2022).

RESULTS

Sample characteristics

A total of 80 cadavers were included in this study, 57 males and 23 females. The mean age of the males and females was 51.5 years (range 24-86 years, standard deviation [SD]=14.23) and 58.3 years (range 29-92 years, SD=20.31), respectively. The mean PMI was approximately 6.9 (SD=3.42) hours in males and 5.6 (SD=3.14) hours in females. Deaths were due to the following causes: sudden cardiac arrest (42.5%), coronary artery disease (31.25%), head and neck injury (6.25%), infectious diseases (6.25%), asphyxia (3.75%), thyrotoxicosis (2.5%), intracerebral hemorrhage (2.5%), senility (2.5%), and gastrointestinal diseases (2.5%).

Using L* and b* colorimetric data from the control area, the cadavers were classified into five types of skin color: very light (7.5%), light (23.75%), intermediate (43.75%), tan (18.75%), and brown (6.25%).

Colorimetric characteristics

Characteristics of color data obtained from the areas of livor mortis before and after application of pressure are illustrated in Figure 2. Values of pressure-induced changes in L*, a*, and b* were analyzed. Lower observed L* values indicated a decrease in the brightness of livor mortis as the postmortem time progressed, while the application of 1.5 kg/cm^2 of pressure made the livor mortis lighter. After 6-hours postmortem, changes in levels of brightness were still slightly positive. Similar to the L* values, the b* value also displayed a general decline over time. As time progressed, the a* values showed an increase in the reddish hue. No statistically significant difference was observed between the left and right scapular areas in any of the L*, a*, and b* data ($p > 0.05$).

Table 1 shows the Pearson correlation coefficients for the relationship between colorimetric measurements (ΔL*, Δa*, Δb*, ΔE*) and PMI. All colorimetric measurements of the control areas showed an insignificant, very weak correlation with PMI (r = -0.07 – 0.22, p = 0.13-0.54). A negative correlation was observed in the L* and b* values before and after application of pressure. In this study, all ΔL*, Δa*, Δb*, and ΔE* were found to be statistically significantly associated with the PMI ($p < 0.01$).

The ΔE* values of the right scapular area before the application of pressure showed the highest correlation with PMI (r = 0.62, $p < 0.01$) and were plotted against postmortem intervals as shown in Figure 3. The regression formula was used to find the rate of change of ΔE* during the measurement interval. Plotting the line of best fit through the points, the relationship between the BP ΔE* values and PMI was found to be as follows: PMI (hours) = 0.388 (ΔE*) + 1.038. However, BP ΔE* had only a weak positive relationship with PMI (adjusted R2 = 0.382, $p < 0.01$), so ROC curves in logistic regression were used to determine the best cut-off values in this dataset.

The area under the ROC curve for the BP ΔE* values was 0.82, 95% CI [0.7-0.9] (Figure 4). The BP ΔE* values were used to discriminate between early PMI (less than 6 hours) and late PMI (6-12 hours). The cut-off value of ΔE* of the right scapular area before the application of pressure, which allows for the best sensitivity and specificity, is 14 (sensitivity 72.5%, specificity 80%) (Table 2). A score range with higher scores indicates a greater likelihood of later PMI.

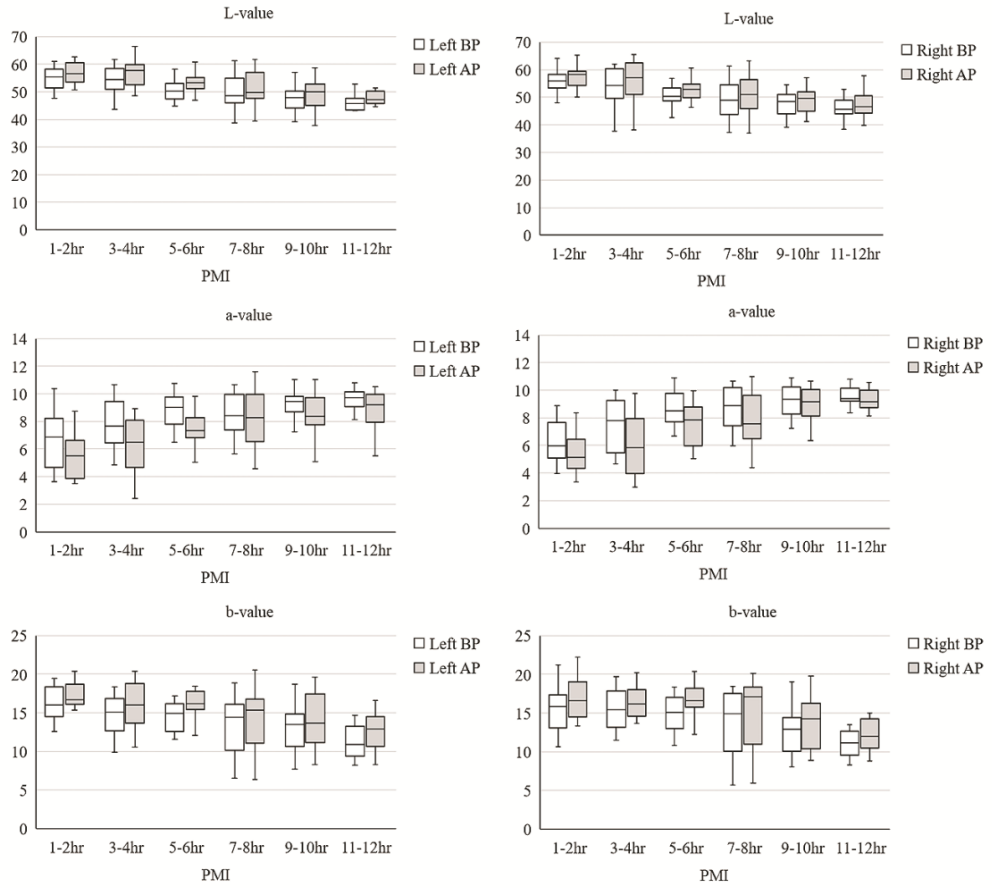

Figure 2. Characteristics of color data obtained from the area of livor mortis before (BP) and after (AP) application of pressure

Table 1. Pearson correlation for PMI and colorimetric measurement before (BP) and after (AP) application of pressure

	Right scapular area								Left scapular area							
	BP				AP				BP				AP			
	ΔL*	Δa*	Δb*	ΔE*	ΔL*	Δa*	Δb*	ΔE*	ΔL*	Δa*	Δb*	ΔE*	ΔL*	Δa*	Δb*	ΔE*
Pearson correlation	-0.54	0.58	-0.49	0.62	-0.52	0.61	-0.47	0.60	-0.53	0.52	-0.50	0.57	-0.53	0.57	-0.46	0.60
p-value	< 0.01															

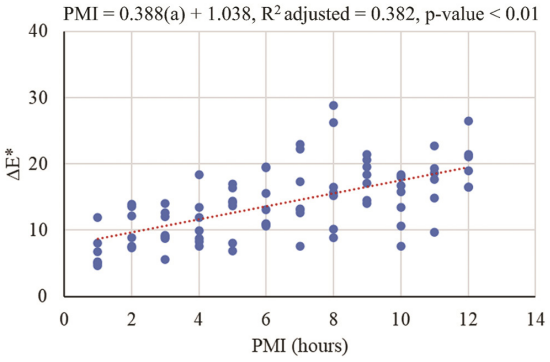

Figure 3. Color difference value (ΔE*) of the right scapular area before applying pressure with different postmortem intervals (PMI)

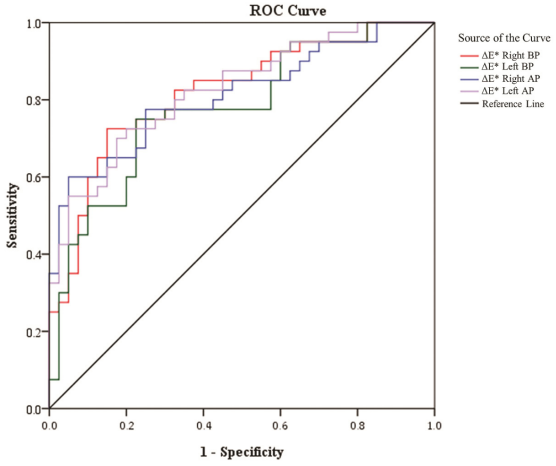

Figure 4. ROC curve associated with the PMI: area under ROC curve of ΔE* values of the right scapular area before application of pressure = 0.82

Table 2. Sensitivity and specificity of ΔE* values of the right scapular area before application of pressure

Cut point (ΔE*)	Sensitivity (%)	Specificity (%)	Accuracy (%)	Positive predictive value (%)	Negative predictive value (%)
12	85	57.5	71.25	66.67	79.31
13	82.5	65	73.75	70.21	78.79
14	72.5	80	76.25	78.38	74.42
15	67.5	85	76.25	81.82	72.34
16	60	87.5	73.75	82.76	68.63

DISCUSSION

In Thailand, livor mortis is commonly used to estimate PMI. Among forensic pathologists, direct observation remains the most popular analytical method for determining livor mortis (4, 7). This study investigated pressure-induced color changes in livor mortis under standardized conditions on a large number of cadavers with known PMI.

A wide variety of skin colors among cadavers were observed. It is considered that the color of the original skin has an impact on the color of postmortem livor mortis (17). Original skin color in this study was determined by the colorimetric measurements of the color of the skin in an area without livor mortis. The results showed a wide variety in the degree of natural pigmentation, ranging from very light to brown, with 43.75% of the cadavers being intermediate in skin color. These findings parallel a skin color study by Del Bino and Bernerd (27), which found the skin color of Caucasians to be generally light to intermediate. The authors of the present study calculated the color difference between postmortem livor mortis and the control skin to standardize the colorimetric parameters before analysis.

Several authors have attempted to use more objective methods to estimate an unknown PMI from livor mortis; however, the wide scattering of outcomes gives the results little practical value in forensic casework (11-17). It is imperative to reconsider the following factors affecting livor mortis: intensity and duration of pressure applied and the area where the pressure is applied. In a recent study of 101 cadavers, Romanelli et al. suggested that a pressure of 1.5 kg/cm^2 and a duration of 3 seconds were the most suitable conditions to make a more standardized analysis of livor mortis (18). The present study attempted to use those parameters to improve the value of livor mortis in the determining the time since death. This study found all colorimetric parameters to be statistically significant ($p < 0.01$), but the data obtained from areas of livor mortis were only weakly correlated with PMI. There is a linear relationship between colorimetric measurements and PMI during the first 12 hours with the degree of color change and brightness associated with livor mortis decreasing as the PMI increases (15). Even though ΔE* showed the highest correlation with the PMI, its regression formula has been reported to have a weak correlation coefficient of 0.382.

A number of issues were considered in conducting this study. The study had to be designed to resemble actual forensic practice, where it is sometimes difficult to find trustworthy information about the history of the deceased and the circumstances of their death. The wide variations in PMI estimation observed in this study can be explained by other variables such as antemortem physical conditions, cause of death, and antemortem and postmortem environmental factors. Due to the variability of the time before each of the remains arrived at the mortuary, environmental factors such as temperature and humidity could have affected the rate of livor mortis development. Such variables need to be considered when determining PMI using colorimetric examination.

In this study, the authors used the ΔE* value from the right scapular area prior to application of pressure to discriminate between early PMI (< 6 hours) and late PMI (6-12 hours). Even though uncontrolled factors such as antemortem status and environmental conditions were inevitably encountered, the authors decided to use a cut-off point with the most appropriate sensitivity and specificity in order to achieve the best possible accuracy. Accordingly, we recommend a cut-off point ΔE* value of 14 to differentiate between early and late PMI.

This study provides quantitative criteria that allow for a more accurate estimation of the time of death. There is a need for additional studies using a larger sample and a wider diversity of skin color characteristics. Postmortem blood concentration may be considered for its role in the formation of livor mortis. Research on how uncontrolled antemortem conditions might affect PMI need to be studied as well. Further research is needed to help develop a better understanding, e.g., studies which include subjects with a known medical history such as the percentage by volume of red cells.

CONCLUSIONS

In forensic medicine, PMI estimation in medicolegal cases is one of the most frequent and challenging topics. Numerous techniques have been developed and implemented in an attempt to improve the accuracy of determination of the PMI. In this study, color change of livor mortis under the application of pressure was evaluated based on colorimetric measurements. By offering objective standards for measuring color change, the method presented here should encourage wider use of pressure-induced blanching of livor mortis in estimating PMI.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

ADDITIONAL INFORMATION

Author contribution

S.N.: conceptualization, methodology, data collection, data analysis, writing - draft. V.V.: supervision, conceptualization, methodology, data analysis, writing – review and editing

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Fetal Anemia in Northern Thailand: Etiologies and Outcomes

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ABSTRACT

OBJECTIVE In Southeast Asia, hemoglobin (Hb) Bart's disease is the primary cause of fetal anemia, although other causes are increasingly being identified. This study aimed to characterize the etiologies and outcomes of fetal anemia in northern Thailand.

METHODS A retrospective chart review was conducted, involving pregnant women who attended antenatal care at Chiang Mai University Hospital between 2014 and 2021 and had a diagnosis by ultrasound findings of fetal anemia, or a fetal diagnosis of Hb Bart's disease or other known hereditary anemias.

RESULTS Among 71 fetuses from 64 pregnancies, 45 (63.4%) had Hb Bart's disease. Twelve cases (16.9%) of fetal anemia were from other causes, including three cases of homozygous Hb Constant Spring, three cases of hereditary pyropoikilocytosis, one case of suspected red cell membrane disorder, one case each of Rh(D) alloimmunization, Hb H/Hb Pakse disease, transient abnormal myelopoiesis, syphilis infection, and one of unknown cause. All of the seven sets of twins (19.7%) had twin-to-twin transfusion syndrome (TTTS). Intrauterine transfusion was given in four cases of fetal hemolytic anemia which rendered good outcomes. Overall, 12 cases (16.9%) survived beyond the neonatal period.

CONCLUSIONS Hb Bart's disease remains the leading cause of fetal anemia in northern Thailand. Increasingly, frequently diagnosed causes include hemoglobinopathies and red cell membrane disorders.

KEYWORDS fetal anemia, Hb Bart's disease, Hb Constant Spring, hydrops fetalis, red cell membrane disorders

INTRODUCTION

Fetal anemia is a significant complication in fetuses. Untreated fetal anemia can lead to hydrops fetalis and fetal demise. Clinical presentations of fetal anemia include fluid collection in the third space, decreased fetal activity, cardiomegaly, hepatosplenomegaly, and an enlarged placenta. Hydrops fetalis commonly develops in advanced cases, characterized by fluid collection in at least two body compartments, and is associated with a high perinatal mortality rate of 50-98% (1, 2).

The etiologies of fetal anemia can be categorized into two groups: immune-mediated and non-immune mediated causes. Rh(D) alloimmunization is the primary cause of immune-mediated fetal anemia in Western populations (3). The use of intravenous anti-Rh(D) immunoglobulin (anti-D Ig) prophylaxis has reduced the incidence of hemolytic disease of the fetus and newborn (HDFN) due to Rh(D) alloimmunization (4). In Southeast Asian populations, Rh(D) alloimmunization is less common due to the low prevalence of the Rh(D)-

negative blood group (1.7%) compared with that in Western populations (17.3%) (5). The leading cause of fetal anemia among Asian populations is hemoglobin (Hb) Bart's disease resulting from homozygous alpha^0-thalassemia (2, 6-8).

In a previous study conducted over a 10-year period in central Thailand, 78 cases of fetal hydrops in stillborns were examined, revealing anemia as the predominant cause (42% of cases), with half of the cases attributed to homozygous alpha^0-thalassemia (9). A recent study of alpha-thalassemia mutations in Southeast Asia, including Thailand, demonstrated a high burden of alpha-thalassemia in Thailand (10). In 2020, 423 new cases of Hb Bart's hydrops fetalis were estimated to have occurred in the country, with the highest absolute burden observed in Bangkok (the capital city) and Udon Thani (a province in the northeastern region). Chiang Mai, the largest city in northern Thailand, also displayed a high prevalence of alpha^0-thalassemia (10). Two large case series conducted in Thailand have consistently identified homozygous alpha^0-thalassemia as the leading cause of hydrops fetalis. Other causes of fetal anemia include homozygous Hb Constant Spring (CS), cardiovascular abnormalities, infections, and red cell membrane disorders (7, 9, 11-13).

Thailand has successfully established a prenatal screening and diagnosis program for couples at risk of severe thalassemia diseases (14). This program offers genetic counseling, prenatal screening, and prenatal diagnosis for couples at risk of having a fetus with severe thalassemia. In cases of homozygous alpha^0-thalassemia, termination of pregnancy is offered to prevent adverse maternal outcomes. The implementation of this program has resulted in a decrease in the number of undetected cases of fetal anemia.

The aim of this study is to examine the causes, treatment strategies, and outcomes of fetal anemia at Chiang Mai University Hospital, a tertiary care hospital in northern Thailand. The results may provide insights into the various diseases that contribute to the occurrence of fetal anemia in the region.

METHODS

A retrospective descriptive study was conducted with the ethical approval of the Institutional Review Board, Faculty of Medicine, Chiang Mai University (Research study ID: PED-2562-06931). The full medical records, including clinical and laboratory information of pregnancies with fetal anemia and/or hydrops fetalis associated with anemia, of patients who were treated at Chiang Mai University Hospital from 2014 through 2021 were comprehensively reviewed. The review process included two steps as follows: First, we screened and retrieved medical records from the hospital for diagnoses potentially associated with fetal anemia and/or hydrops fetalis, including ICD-10 (International Statistical Classification of Diseases and Related Health Problems, 10th Revision) codes O361-363, P50, P55, P56, P613, P614, and P832. After that, the confirmed cases were reviewed in detail.

Fetal anemia was defined based on one or more of the following criteria: ultrasound findings of middle cerebral artery-peak systolic velocity (MCA-PSV) greater than 1.5 MoM, other ultrasound findings indicating fetal anemia, fetal hematocrit levels below 30%, and fetal diagnosis through Hb analysis or molecular methods indicating homozygous alpha^0-thalassemia (14). Hydrops fetalis was defined as the presence of excessive fluid accumulation in two or more body cavities. Demographic, clinical, and laboratory data of the mothers, fetuses and newborns were validated, recorded and analyzed.

Frequencies are presented as numbers and percentages. Continuous data is presented as medians plus interquartile range (IQR) or mean ± standard deviation, according to the normality of the data distribution. Statistical analysis was performed using SPSS Statistics for Windows, (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY, USA, IBM Corp.).

RESULTS

A total of 1,755 medical records were retrieved and screened, revealing 71 fetuses from 64 pregnancies diagnosed with fetal anemia and/or hydrops fetalis associated with anemia. Table 1 presents the clinical characteristics, etiologies, and outcomes of these cases. Of all the cases, 57 (80.3%) were diagnosed with fetal anemia, and 14 (19.7%) had fetal anemia/volume overload from twin-to-twin transfusion syndrome (TTTS). Table 2 provides the clinical characteristics and outcomes categorized by etiology.

Table 1. Clinical characteristics, etiologies, and outcomes of 64 pregnancies (71 fetuses) with a diagnosis of fetal anemia

Clinical characteristics	Results
Maternal age (median, IQR, year)	28.5 (11.0)
Gestational age at diagnosis (median, IQR, week)	20 (7)
Gestational age at delivery (median, IQR, week)	21 (9)
Birth weight (median, IQR, g)	450 (895)
Diagnosis	
Fetal anemia (N, %)	
- Hb Bart's disease (homozygous alpha0-thalassemia)	45 (63.4%)
- Hemolytic anemia	9 (12.7%)
- Syphilis infection	1 (1.4%)
- Transient abnormal myelopoiesis	1 (1.4%)
- Anemia, unknown cause	1 (1.4%)
Fetal anemia/volume overload (N, %)	
- Twin-twin transfusion syndrome	14 (19.7%)
Outcomes (N, %)	
- Termination of pregnancy	48 (67.6%)
- Fetal demise *in-utero*	8 (11.3%)
- Neonatal death	3 (4.2%)
- Survival beyond neonatal period	12 (16.9%)

Table 2. Clinical characteristics and outcomes of 64 pregnancies (71 fetuses) with a diagnosis of fetal anemia as classified by etiology

Clinical characteristics	Fetal anemia					Fetal anemia/ volume overload
	Hb Bart's disease (N = 45)	Hemolytic anemia (N = 9)	Syphilis infection (N = 1)	Anemia (unknown cause) (N = 1)	Transient abnormal myelopoiesis (N = 1)	Twin-twin transfusion syndrome (N = 14)
Maternal age (median, IQR, year)	31 (9)	23 (14)	23	22	40	29 (7)
Gestational age at diagnosis (median, IQR, week)	18 (9)	24.5 (11)	23	22	25	25 (6)
Gestational age at delivery (median, IQR, week)	20 (7)	33 (11)	23	26	33	27.5 (8)
Birth weight (median, IQR, g)	300 (415)	1,833 (1,781)	875	NA	1,985	1,145 (1,141)
Treatment with intrauterine transfusion (N, %)	-	4 (44.4%)	-	-	-	-
Outcomes (N, %)						
- Termination of pregnancy	45 (100%)	1 (11.1%)	-	-	-	2 (14.3%)
- Fetal demise inutero	-	1 (11.1%)	1 (100%)	1 (100%)	-	5 (35.7%)
- Neonatal death	-	1 (11.1%)	-	-	1 (100%)	1 (7.1%)
- Survival beyond neonatal period	-	6 (66.7%)	-	-	-	6 (42.9%)

Hb Bart's disease

The most common cause identified was Hb Bart's disease resulting from homozygous Southeast Asian deletional alpha^0-thalassemia, accounting for 63.4% (45 out of 71 cases). Of those, 42 were diagnosed through the prenatal screening and diagnosis program, whereas the remaining three cases with incomplete screening were detected by prenatal sonographic signs of fetal anemia in the second trimester. Among the 45 pregnancies, 32 couples opted for prenatal diagnostic techniques such as chorionic villi sampling,

amniocentesis, or cordocentesis to confirm the diagnosis of Hb Bart's disease. Thirteen couples chose serial ultrasound monitoring, all of which developed hydropic changes, leading to invasive prenatal diagnosis for confirmation. Common ultrasound findings in this group included cardiomegaly (53.3%), high MCA-PSV (44.4%), and pericardial effusion (28.9%). The median (IQR) gestational age at diagnosis was 16 (6.5) weeks for the prenatal diagnostic test group and 22 (4.5) weeks for the serial ultrasound group. After counseling, all pregnancies affected by Hb Bart's disease were terminated.

Hemolytic anemias other than Hb Bart's disease

Nine fetuses were diagnosed with hemolytic anemia other than Hb Bart's disease, including three cases of homozygous Hb CS, three cases of hereditary pyropoikilocytosis (HPP) caused by homozygous or compound heterozygous *SPTB* or *SPTA1* mutations as identified by whole exome sequencing analysis (11), and 1 case each of Hb H/Hb Pakse disease, unidentified red cell membrane disorder, and Rh(D) alloimmunization. The diagnosis of unidentified red cell membrane disorder in one fetus was made based on findings of abnormal red cell morphology in the fetus and the parents; molecular analysis was not performed in this family. All fetuses exhibited signs of anemia and/or hydrops fetalis in the second or third trimesters. Common ultrasound findings in this group were cardiomegaly (66.7%), high MCA-PSV (55.6%), pericardial effusion (55.6%), and ascites (44.4%). One fetus diagnosed with HPP was terminated, while one fetus with an unidentified red cell membrane disorder died in utero. One fetus with HPP died during the neonatal period. Intrauterine transfusion was administered to four fetuses: one with Rh(D) alloimmunization, one with Hb H/Hb Pakse disease, and two with homozygous Hb CS. One fetus with homozygous Hb CS and fetal anemia did not receive intrauterine transfusion due to a parental decision against invasive prenatal interventions. Ultrasonographic evaluation at 21 weeks of gestational age revealed high MCA-PSV and fetal cardiomegaly. Regular monitoring with ultrasonography demonstrated resolution of anemia signs by 32 weeks of gestation. The patient was delivered at 39 weeks of gestation. He had congenital pneumonia necessitating mechanical ventilation for a period of two days. At birth, hemoglobin and hematocrit levels were measured at 13.9 g/dL and 44.3%, respectively, with no requirement for red blood cell transfusion during the neonatal period.

The patient group with hemolytic anemias other than Hb Bart's disease had the highest perinatal survival rate (66.7%), with 6 surviving fetuses. Among the survivors, only the one case with HPP remained transfusion-dependent. Despite receiving intrauterine transfusion and anti-D immunoglobulin, the newborn with Rh(D) alloimmunization experienced perinatal complications of severe hemolysis requiring partial exchange transfusion and jaundice from inspissated bile syndrome. Three newborns with homozygous Hb CS and one with Hb H/Hb Pakse disease survived beyond the neonatal period. Table 3 shows the clinical characteristics and treatment received during neonatal period of the six surviving fetuses in this group.

Anemia from other causes

In the fetal anemia group, apart from hemolytic anemia, there was one fetus with trisomy 21 and transient abnormal myelopoiesis (TAM) who died during the neonatal period, one fetus with syphilis infection, and one with a condition of unknown cause. The latter two fetuses died in utero.

Twin-to-twin transfusion syndrome (TTTS)

TTTS was observed in 14 fetuses of 7 pregnancies. Initial ultrasound findings included ascites (50.0%), generalized skin edema (42.8%), cardiomegaly (35.7%), and pericardial effusion (28.6%). TTTS was diagnosed in the second or third trimesters. None received laser coagulation. This group had the second highest perinatal survival rate (42.9%).

DISCUSSION

Hb Bart's disease or homozygous alpha^0-thalassemia remains the most prevalent cause of fetal anemia in northern Thailand, indicating a high gene frequency of alpha^0-thalassemia in the population. The leading diagnosis of Hb Bart's disease in our study is consistent with that reported in previous studies in Thailand and China (2, 7, 13, 15). Increasingly, diagnosed causes of fetal anemia are non-deletional alpha-thalassemia and hereditary

Table 3. Clinical characteristics and treatment received during neonatal period of six surviving fetuses in the group of hemolytic anemias other than Hb Bart's disease

Case number	Diagnosis	GA at onset of anemia	GA at delivery	Birth weight (g)	Intrauterine transfusion (times)	Hb before IUT (g/dL)	Hb at birth (g/dL)	Treatment received during neonatal period
1	Rh alloimmunization	32	34	2,210	1	4.2	6.1	Red cell transfusion Phototherapy Exchange transfusion
2	Hereditary pyropoikilocytosis	30	30	1,455	0	3.6	4.5	Red cell transfusion Phototherapy Exchange transfusion
3	Hb H/Hb Pakse disease	18	37	2,860	1	6.5	17.2	Phototherapy
4	Homozygous Hb Constant Spring	21	39	2,805	0	not done	13.9	Ventilator support for congenital pneumonia
5	Homozygous Hb Constant Spring	25	N/A	N/A	1	N/A	N/A	N/A
6	Homozygous Hb Constant Spring	22	40	3,310	1	4.7	15.0	Phototherapy

*GA, gestational age; Hb, hemoglobin; IUT, intrauterine transfusion; N/A, data not available

red cell membrane disorders. Of note, only one fetal anemia associated with Rh(D) alloimmunization was identified in this study, suggesting a low prevalence of the Rh(D)-negative blood group in the Thai population.

Hb CS is a commonly observed Hb variant in Southeast Asian populations (16). Hb CS results from a nucleotide substitution at the termination codon of the alpha-2 globin gene, *HBA2*:c.427 T>C. This substitution replaces the termination codon with glutamine, resulting in an elongated and unstable alpha-globin variant (17). The compound heterozygosity of alpha^0-thalassemia and Hb CS results in Hb H/Hb CS disease, which typically presents with a moderate degree of chronic hemolytic anemia. Some patients may require regular transfusion for an extended period. In older children and adults with homozygous Hb CS, mild non-transfusion-dependent chronic hemolytic anemia is commonly observed. However, homozygous Hb CS has been reported as a cause of fetal anemia that shows a good response to treatment and a good long-term outcome (12, 18, 19). These fetuses may present with severe anemia necessitating intrauterine transfusion. Nevertheless, in late gestation, hemolysis tends to decrease, possibly because of Hb switching process, resulting in lower Hb F and higher Hb A levels. The anemia becomes milder after birth. Considering the potential impact of homozygous Hb CS, it is crucial to include it in the differential diagnosis of fetal anemia in Southeast Asian populations. Early diagnosis and intervention can contribute significantly to achieving a favorable outcome. In this study, fetuses with homozygous Hb CS and Hb H/Hb Pakse who received intrauterine transfusion were born at term with a normal Hb level and did not require red cell transfusion during the neonatal period.

Hb H disease results from mutations affecting three out of four functioning alpha-globin alleles, leaving one intact alpha-globin allele. Hb H disease can be classified as either deletional (genotype --/-α) or non-deletional (genotype --/α^{T}α or --/α α^T) Hb H disease. Generally, patients with Hb H disease present with a mild to moderate degree of anemia. Patients with non-deletional Hb H disease experience more severe anemia and may be transfusion dependent. Hb H hydrops fetalis represents the most severe form of Hb H disease, characterized by severe fetal anemia. Mutations causing Hb H hydrops fetalis typically involve alpha^0-thalassemia on one chromosome and a non-deletional mutation causing a hyperunstable or unstable Hb on the other chromosome (20, 21). Previously reported genotypes of common Hb H

disease that can be associated with Hb H hydrops fetalis are Hb H/Hb CS disease and Hb H/Hb Pakse diease (22, 23). One case in this study was diagnosed with Hb H/Hb Pakse disease.

Red cell membrane disorders are a frequently diagnosed cause of fetal severe hemolytic anemia (11, 24). The availability of next-generation sequencing has facilitated the identification of causative mutations and related genes. In this study, three cases of HPP were identified, indicating a high prevalence of HE in the population. Accurate molecular diagnosis of HPP is essential for treatment planning and genetic counseling.

TTTS is a potentially life-threatening complication that occurs in 10-15% of monochorionic twin pregnancies (25). This condition arises from an imbalance of blood flow between the placental anastomoses. Hydrops fetalis typically develops in the donor fetus due to anemia and high-output heart failure. Signs of heart failure include subcutaneous edema, ascites, and pericardial to pleural effusion prior to the development of hydrops fetalis (26, 27).

Diagnosis of TTTS is based on ultrasound findings (28). To facilitate early detection, it is recommended that biweekly ultrasounds be performed after 16 weeks of gestational age for monochorionic twins to evaluate fetal growth and amniotic fluid volume. MCA-PSV and umbilical artery flow pattern should be assessed after 20 weeks of gestational age (29). Fetoscopic laser coagulation is the standard treatment for TTTS between 17 and 26 weeks of gestational age and is intended to interrupt the anastomoses. This intervention leads to survival rates of approximately 70% for both twins and at least 90% for pregnancies with at least one survivor (30).

Of note, the compilation of the ICD-10 codes used in this study was designed to identify as many fetal anemia cases as possible. A total of 1,755 medical records were screened, but only 71 fetuses were enrolled. A large number of cases were excluded due to overlaps with diagnoses of other common conditions such as neonatal hyperbilirubinemia, non-anemic hydrops fetalis resulting from other causes and anemia with neonatal onset. Limitations of this study include the lack of long-term neurodevelopmental evaluation in perinatal survivors. Additionally, due to the retrospective nature of the study, some medical records may not have been entirely reliable, potentially resulting in under-diagnosis of fetal anemia and so may have been missed during the review.

CONCLUSIONS

In conclusion, this study demonstrates that Hb Bart's disease remains the primary cause of fetal anemia in the northern Thai population. Homozygous Hb CS, Hb H hydrops fetalis, and red cell membrane disorders are increasingly diagnosed causes of fetal anemia. Intrauterine transfusion has been proven to be beneficial in cases of homozygous Hb CS.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

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Knowledge and Awareness of Human Papillomavirus Infection and Vaccination in Thai Male Youth, Including Men Who Have Sex with Men

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ABSTRACT

OBJECTIVE There is currently a lack of human papillomavirus (HPV) vaccination policy and education for male youth in Thailand. This study aimed to evaluate Thai male youth's knowledge and awareness of HPV infection and vaccination, determine their HPV vaccination rate, and factors related to the vaccination rate and the level of awareness of HPV.

METHODS A questionnaire survey on HPV vaccination was distributed to educational institutions across different regions of Thailand. Inclusion criteria included Thai male youth aged between 15 and 24 years, regardless of sexual orientation. Exclusion criteria were inability to access the internet, individuals whose responses were unintelligible, and those who did not complete the questionnaire. The trends of the association between participant characteristics and their HPV knowledge/awareness scores was analyzed using linear regression.

RESULTS A total of 594 individuals responded to the questionnaire. The median score for knowledge was 11 out of 18 and the awareness level was 80%. Higher education level, higher family income, bisexuality, and prior receipt of HPV information were statistically significantly linked to higher HPV knowledge scores. However, only previous receipt of HPV information was associated with an increased awareness level.

CONCLUSIONS Although the level of HPV knowledge and awareness among Thai males was acceptable, less than 50% of participants expressed an intention to get vaccinated. This indicates there is a need to improve the promotion of the HPV vaccine in order to achieve herd immunity.

KEYWORDS HPV knowledge, HPV vaccines, men who have sex with men, vaccination intention

INTRODUCTION

Human papillomavirus (HPV) infection is one of the most common sexually transmitted diseases (STDs) in both males and females (1). In fact, as many as 62.5% of men have been found to have asymptomatic HPV infections in their genitalia. Most of these cases are caused by HPV type 16 (2). High-risk HPV, particularly types 16 and 18, is the primary cause of HPV-related cancers, e.g., anal, penile, head and neck, and oropharyngeal cancers, which are transmitted primarily through sexual activity. Although cervical cancer screening programs can help detect HPV-related gynecologic cancer in women, there are currently no comparable screening methods available for detecting HPV-related cancer in men. Approximately 75%

of women with HPV-positive sexual partners have HPV DNA in their cervix (3). It's worth noting that males who engage in sexual activities with men (MSM) are at increased risk of contracting HPV. Studies have shown that anal HPV infection has a prevalence of 58.5% among MSM, and high-risk HPV infection has a prevalence of 36.6%. Therefore, it's important to take preventive measures and follow safe sex practices to reduce the risk of HPV transmission (4). According to the latest data, a staggering 86.6% of high-risk HPV-infected MSM in Northern Thailand have developed cancerous lesions. This highlights the urgent need for more effective prevention and early detection measures to tackle this important issue (5).

It is known that HPV vaccines are equally safe and effective for both males and females (6). According to available data, HPV vaccines are effective in preventing HPV-associated diseases in men, including MSM. Studies have shown that in males who have received the vaccine, there is an 86% reduction in persistent genital HPV infection and a 90% decrease in the incidence of external genital lesions (7). After HPV vaccination, a significant drop in anogenital warts was reported in both men and women in a country where vaccination coverage was at least 50% in women aged 13-19 years, suggesting herd immunity (8). In the US, the HPV vaccine results in $1.8 billion in direct medical costs annually (9). The incidence of HPV-related anal and oropharyngeal cancers has been increasing recently among men, and is remarkably high (20 times higher) among MSM in the US (9). The rates are lower among MSM who have received prophylactic HPV vaccination. According to a recent study, after an HPV vaccination program for men was introduced in the US in 2011, vaccine coverage has grown to 17.2%. However, despite this progress, the vaccination rate is still considered low (10).

In Thailand, the Ministry of Public Health has subsidized HPV vaccines for all fifth-grade female students in a national vaccination program since 2017 (11). However, health care benefits provided by the Civil Servant Medical Benefit Scheme (CSMBS), Social Security scheme (SSS), and the Universal Coverage program (UC) do not currently include men in the HPV vaccine program. In order to achieve maximum protection for the population, it is recommended that men as well as women receive the HPV vaccine. This could aid in reaching the necessary herd immunity level of 70-80% coverage, which, in turn, would help reduce the spread of the virus and decrease the occurrence of related health issues (12).

CDC guidelines recommend that females aged 9-14 i.e., those of adolescent age, receive HPV vaccine. In Thailand, the recommended age for vaccination is 9 to 26 years old. The number of antibodies produced after HPV vaccination decreases with age and significantly declines after first sexual intercourse (13). Although there is no national data, studies have found that Northern Thai teenagers have their first sexual intercourse on average at age 16.7 years, when they have the highest risk of getting HPV infection (14). Because this group is a non-obligatory population for HPV immunization even though they are a cog in the vicious disease cycle, they should be assessed regarding their knowledge and awareness of the topic.

This study is centered on Thai male youth between the ages of 15 and 24 years from various regions in Thailand. Its objective is to explore their awareness of HPV infection as well as their knowledge of HPV vaccination, which together reflect the effectiveness of public health promotion efforts. This study also seeks to identify factors that influence the decision of men to get HPV vaccination to help guide future promotion of HPV-related disease prevention efforts.

METHODS

Study design and participants

The design is a cross-sectional study of the Thai male youth population aged between 15 and 24 years, regardless of sexual orientation. The exclusion criteria are inability to access the internet, provision of illegible answers and failure to complete the questionnaire. The sample size calculation, based on the Thai male youth population aged 15-24 years from the National Statistical Office in 2019 (a total of 4,421,449), to gain 95% confidence interval and 5% margin of error, indicated a necessary sample size of 385.

The data on knowledge and awareness of HPV plus intention to be vaccinated against HPV infection was collected from August to October 2021 through a questionnaire. The survey was created using Google Form and sent to participants via

a QR code link. The QR code was distributed to educational institutions which had been selected based on the target population group in each region using stratified randomization. After selecting the institutions, direct letters containing advertising posters and QR codes were sent to each high school and vocational school, requesting their help in publicizing the survey. However, this method could not be used for university students due to the large number of students and the separation between student and faculty communities. For university students, project information and QR codes were posted on the main Facebook® group of each of the randomly selected institutions.

A Google form-based cross-sectional survey questionnaire consisting of 3 sections adapted from Dany et al. and Villanueva et al. (15, 16) was used. The questionnaire was translated into the Thai language, and was validated by Dr. Jatupol Srisomboon, a specialist in HPV in Thailand. The first section explored participants' demographics, including age, sexual orientation, sexual history, religion, family income, educational level, address region, and sources of receiving information. Section 2 contained 23 questions and investigated knowledge and awareness of HPV infection and vaccination. The first 18 questions of this section were true or false questions to determine the participants' general knowledge about HPV infection and vaccination. The remaining five questions were used to assess participants' agreement with specific statements using a 5-point Likert scale ("Strongly Disagree" to "Strongly Agree"). The last section asked both vaccinated and unvaccinated participants for their HPV vaccination status, their intention to receive HPV vaccination for those currently unvaccinated, and the rationale behind their decision. Each email account was allowed to respond only once to avoid repetitive responses.

Ethical considerations

At the outset of the questionnaire, the participants were informed that their involvement was completely voluntary. Certain questions, such as those pertaining to gender and sexual orientation, were sensitive, and the participants were told they had the right to choose not to answer them and that all raw data collected would be kept confidential and reported only as analyzed data. The participants' identities were not collected; however, a phone number was gathered for each participant solely as a means of contacting them at the end of the project. The phone number information was kept separate from the research data and to maintain confidentiality was only visible to the researchers.

Ethics approval and consent to participate

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted following the Declaration of Helsinki, and the protocol was approved by the Faculty of Medicine, Research Ethics Committee (OBG-2564-07878).

Data analysis

Our analysis indicated that the missing data in our study were missing at random (MAR), primarily because of the likelihood that missing data for certain variables was related to other observed data rather than to the missing data itself.

Given this MAR pattern, we chose to conduct a complete case analysis for each outcome. This decision was based on the nature of the missing data, which did not significantly skew our sample's representativeness. While we considered more complex methods such as multiple imputation, the MAR assumption and a subsequent sensitivity analysis confirmed that our approach did not compromise the study's findings.

Data are presented as percentages for categorical variables and median with IQR for non -normal distribution continuous variables. Linear regression was used for the trend of association between variables of interest (age, sexual orientation, sexual history, educational level, address region, religion, economic status, having heard of HPV infection and vaccination status before the survey) and HPV knowledge/awareness score. Multivariable analysis was performed using multiple linear regression using the enter method to identify significant factors associated with knowledge and awareness of HPV infection and vaccination. The respondent's rationale behind the decision in both the vaccinated and non-vaccinated groups was collected. Variables correlated with intention to get HPV vaccination in the future were investigated using the Chi-square test. Stata® version 15 and Jamovi® were used for statistical analysis in this study. $P < 0.05$ was considered statistically significant.

Knowledge score

For each question answered correctly, the participants were given one point, while incorrect answers and responses of "do not know" received zero points. The total number of points earned by each participant was calculated, and the average score was used to determine the level of knowledge demonstrated. Those with higher scores were considered to have a greater level of knowledge.

Awareness score

Participants' awareness scores were calculated based on their responses to five statements using a 5-point Likert scale (1=strongly disagree, 5= strongly agree). The scores are presented as a percentage, with higher scores indicating greater awareness.

RESULTS

A total of 614 participants participated in the survey of whom 20 did not complete the survey and were excluded from the analysis. Table 1 presents the participants' demographic data, including sexual history, as well as their level of knowledge and awareness of HPV infection and vaccination.

The median age of the participants was 19 years, with an interquartile range (IQR) of 16 to 21 years. Nearly half of the participants (49.8%) were university students. Regarding the participants' geographic location, almost 40% resided in the Central region. The survey also found that the majority of respondents (90.1%) identified as Buddhists, while the remaining 10% identified as atheists, Christians, or Muslims. In terms of family income, the majority (34.3%) fell within the range of 25,001 to 50,000 baht per month, with the second-highest group (21.4%) earning less than 25,001 baht per month. Almost 30% belonged to the upper-middle- to high-income group (earning more than 75,000 baht per month).

Regarding sexual orientation and behavior, a third of the participant had had sex; the median age of sexual debut was 18 (IQR 16-19). The majority of the Thai male youth were heterosexual (74.4%), while the rest were homosexual (14.1%), bisexual (8.8%), or others, e.g., asexual (1.4%). Regarding receipt of information about HPV infection or vaccination, 316 of 594 participants (53.2%) had heard about HPV or the HPV vaccine.

In the second part of the survey, participants were assessed regarding their knowledge and awareness about HPV infection and vaccination. The median score for knowledge was 11 out of 18 (IQR 7-13). The median awareness score for HPV infection was 80% (IQR 68-92). Finally, the participants were asked about their HPV vaccination status. Out of all the participants, only 25 (4.2%) had received the HPV vaccine, while the remaining 95.8% were unsure or had not yet received it.

HPV infection and vaccination knowledge

Details of questions in the questionnaire and participants' responses are shown in Supplementary Table 1 (Table S1). About 462 (77.8%) of the participants correctly answered that HPV could be transmitted to a sexual partner regardless whether they were male or female, and 434 participants (73.1%) knew that men could receive HPV vaccine. Although 376 (63.3%) and 275 (46.3%) of the participants correctly responded that HPV infection increases the risk of penile cancer and anorectal cancer, respectively, only 188 participants (31.6%) knew that HPV infection increases the risk of nasopharyngeal cancer.

Uni- and multivariable linear regression analysis was used to identify factors significantly associated with HPV infection and vaccination knowledge (Table 2). In univariable analysis, factors that tended to be associated with a higher knowledge score included older age ($p < 0.001$), university educational level ($p = 0.004$) and high vocational certificates ($p = 0.038$), and family income of more than 50,000 baht/month ($p < 0.05$). Also, bisexuals tended to have higher knowledge scores than heterosexuals ($p = 0.004$). In addition, participants who had heard about HPV tended to have a higher knowledge score than those who had not ($p < 0.001$).

In the multivariable analysis that considered age, education, family income, sexual orientation, and receipt of information regarding HPV, participants studying for high vocational certificates had significantly higher knowledge scores than those in junior high school. Family incomes of between 50,001-75,000 and more than 100,000 baht/month were significantly associated with higher knowledge scores compared with family incomes less than 25,000 baht/month. Participants with a family income of 75,001-100,000 tended to have

Table 1. Demographics, including sexual-related history, and HPV knowledge and awareness of participants

Characteristics	N (%) or Median (IQR)
Age (N=594)	19 (16-21)
Education (N=594)	
Junior high school	54 (9.1)
High school	170 (28.6)
University	296 (49.8)
Vocational certificate	44 (7.4)
High vocational certificate	29 (4.9)
Non-formal education	1 (0.2)
Region (N=594)	
Central	231 (38.9)
North	108 (18.2)
Northeast	177 (29.8)
South	62 (10.4)
East	13 (2.2)
West	3 (0.5)
Religion (N=594)	
Buddhism	535 (90.1)
Christianity	15 (2.5)
Islam	3 (0.5)
Atheist	41 (6.9)
Family income (baht per month) (N=505)	
Less than 25,000	108 (21.4)
25,000-50,000	173 (34.3)
50,001-75,000	75 (14.9)
75,001-100,000	61 (12.1)
More than 100,000	88 (17.4)
History of sexual intercourse (N=566)	
No	379 (67.0)
Yes	187 (33.0)
Age of first sexual intercourse (N=594)	18 (16-19)
Sexual orientation (N=563)	
Heterosexual	419 (74.4)
Homosexual	84 (14.9)
Bisexual	52 (9.2)
Others	8 (1.4)
People heard about HPV infection and vaccination before the survey (N=594)	
No	278 (46.8)
Yes	316 (53.2)
HPV infection and vaccination knowledge (full score 18) (N=594)	11 (8-13)
HPV infection and vaccination awareness (%) (N=594)	80 (68-92)
HPV vaccination status (N=594)	
Yes	25 (4.2)
No	490 (82.5)
Unsure	79 (13.3)

higher knowledge scores than those with incomes less than 25,000 baht/month, but the difference was just short of statistical significance (p = 0.053). Lastly, bisexuality and receipt of HPV information were significantly associated with both a higher incidence of HPV infection and higher vaccination knowledge scores than both heterosexuality and never having heard about HPV.

HPV infection and vaccination awareness

A total of 594 participants responded to the five questionnaire items related to their awareness of HPV and the HPV vaccine. Responses

Table 2. Association between characteristics of Thai male youth and HPV infection and vaccination knowledge

Characteristics	Univariable analysis				Multivariable analysis			
	Coefficient (β)	95% Confidence Interval		*p*-value	Coefficient (β)	95% Confidence Interval		*p*-value
		Lower	Upper			Lower	Upper	
Age (N=594)	0.31	0.18	0.44	< 0.01**				
Education (N=594)								
Junior high school (N=54)	Ref.				Ref.			
High school (N=170)	-0.02	-1.34	1.29	0.97	0.70	-0.67	2.07	0.32
University (N=296)	1.84	0.60	3.09	< 0.01**	1.42	-0.77	3.60	0.20
Vocational certificates (N=44)	-0.32	-2.03	1.39	0.71	-0.15	-2.03	1.72	0.87
High vocational certificates (N=29)	2.05	0.12	4.00	0.04*	3.06	0.74	5.37	0.01**
Non-formal education (N=1)	5.02	-3.46	13.50	0.25				
Region (N=594)								
Central (N=231)	Ref.							
North (N=108)	-0.21	-1.21	0.80	0.69				
Northeast (N=177)	-0.63	-1.49	0.23	0.15				
South (N=62)	-0.36	-1.59	0.87	0.56				
East (N=13)	-1.05	-3.50	1.40	0.40				
West (N=3)	-3.28	-8.28	1.71	0.20				
Religion (N=594)								
Buddhism (N=535)	Ref.							
Christianity (N=15)	0.28	-1.97	2.53	0.81				
Islam (N=3)	-0.26	-5.23	4.73	0.92				
Atheist (N=41)	0.74	-0.66	2.13	0.30				
Family income (bath per month) (N=505)								
Less than 25,000 (N=108)	Ref.				Ref.			
25,000-50,000 (N=173)	0.53	-0.49	1.56	0.31	0.42	-0.66	1.51	0.44
50,001-75,000 (N=75)	1.83	0.57	3.09	< 0.01**	1.44	0.14	2.74	0.03*
75,001-100,000 (N=61)	1.41	0.07	2.75	0.04*	1.36	-0.02	2.73	0.05*
More than 100,000 (N=88)	1.93	0.73	3.14	< 0.01**	1.49	0.23	2.75	0.02*
History of sexual intercourse (N=566)								
No (N=379)	Ref.							
Yes (N=187)	0.48	-0.29	1.25	0.22				
Age of first sexual intercourse (N=594)	0.05	-0.03	0.13	0.24				
Sexual orientation (N=563)								
Heterosexual (n=52)	Ref.				Ref.			
Homosexual (n=419)	1.00	-0.02	2.02	0.05	0.60	-0.39	1.60	
Bisexual (n=84)	1.83	0.58	3.09	< 0.01**	1.53	0.30	2.75	0.24
Others (n=8)	0.81	-2.23	3.86	0.60	-0.38	-3.12	2.37	0.01*
Previous receiving information about HPV infection and vaccination (N=594)								0.79
No (n=278)	Ref.				Ref.			
Yes (n=316)	3.72	3.08	4.36	< 0.01**	3.39	2.65	4.12	<0.01**

*, $p < 0.05$; **, $p < 0.01$

were rated on a 5-point Likert scale. The overall awareness score was found to be high, with a median of 80% and an interquartile range (IQR) between 68% and 92%. More detailed information about the individual questions, including participants' opinions about HPV infection and vaccinating males, is shown in Supplementary Table 2 (Table S2). Interestingly, approximately 28% of the Thai male youths did not believe that they were at risk of HPV infection or that they needed the HPV vaccine.

The study included an analysis of the factors associated with awareness of HPV infection and vaccination, similar to that for the knowledge

Table 3. Association between characteristics of Thai male youth and HPV infection and vaccination awareness

Characteristics	Univariable analysis				Multivariable analysis			
	Coefficient (β)	95% Confidence interval		*p*-value	Coefficient (β)	95% Confidence interval		*p*-value
		Lower	Upper			Lower	Upper	
Age (N=594)	0.84	0.42	1.26	< 0.01**				
Education (N=594)								
Junior High school (n=54)	Ref.							
High school (n=170)	2.25	-2.14	6.65	0.31				
University (n=296)	6.73	2.57	10.89	< 0.01**				
Vocational certificates (n=44)	3.89	-1.82	9.60	0.18				
High vocational certificates (n=29)	4.20	-2.27	10.67	0.20				
Non - formal Education (n=1)	12.89	-15.49	41.26	0.37				
Region (N=594)								
Central (n=231)	Ref.							
North (n=108)	0.38	-2.93	3.69	0.82				
Northeast (n=177)	-2.12	-4.95	0.72	0.14				
South (n=62)	1.18	-2.88	5.25	0.57				
East (n=13)	-4.48	-12.58	3.62	0.28				
West (n=3)	-2.84	-19.35	13.67	0.74				
Religion (N=594)								
Buddhism (n=535)	Ref.							
Christianity (n=15)	-2.11	-9.55	5.32	0.58				
Islam (n=3)	12.55	-3.88	28.99	0.13				
Atheist (n=41)	2.41	-2.19	7.01	0.31				
Family income (bath per month) (N=505)								
Less than 25,000 (n=108)	Ref.							
25,000-50,000 (n=173)	0.08	-3.33	3.47	0.97				
50,001-75,000 (n=75)	0.31	-3.86	4.48	0.88				
75,001-100,000 (n=61)	3.187	-1.25	7.63	0.16				
More than 100,000 (n=88)	1.63	-2.35	5.61	0.42				
History of sexual intercourse (N=566)								
No (n=379)	Ref.							
Yes (n=187)	2.94	0.40	5.48	0.02*				
Age of first sexual intercourse (N=187)	0.02	-0.22	0.26	0.89				
Sexual orientation (N=563)								
Heterosexual (n=52)	Ref.							
Homosexual (n=419)	4.08	0.67	7.49	0.02*				
Bisexual (n=84)	2.73	-1.47	6.93	0.20				
Others (n=8)	5.27	-4.92	15.46	0.31				
Previous receiving information about HPV infection and vaccination (N=594)								
No (n=278)	Ref.				Ref			
Yes (n=316)	6.43	4.15	8.71	< 0.01**	6.08	3.56	8.60	< 0.01**

*, $p < 0.05$; **, $p < 0.01$

scores (Table 3). The analysis was performed using both uni- and multivariable linear regression. The analysis identified several determinants that were statistically significant in the univariable analysis. For example, the analysis revealed that individuals who were older and had higher education levels, e.g., university education, had a greater awareness of HPV infection and vaccination than those who were younger and had lower education levels, e.g., a junior high school education. The study also found that participants who had already engaged in sexual intercourse or had heard about HPV were more aware of HPV infection. Furthermore, the study highlighted the importance of sexual orientation as a factor related to awareness, as individuals who identified

as homosexual tended to be more aware of HPV infection and vaccination compared to those who identified as heterosexual. The variables which showed statistical significance ($p < 0.05$) in the univariate analysis, e.g., age, education, history of sexual debut, and previous receipt of HPV information, were further assessed using multivariable linear regression analysis. However, only the variable related to previous receipt of HPV information maintained its significance ($p < 0.001$) in the multivariable analysis.

Rationale behind decisions regarding HPV vaccination

According to the survey, only 4.2% of young Thai males have received the HPV vaccination. Among those who had been vaccinated, most made the decision to do so on their own, followed by parental suggestions, healthcare worker recommendations, and encouragement from friends. The present study also explored reasons why some participants did not receive the HPV vaccine. The most common reason was that they were not aware that men could receive the HPV vaccine (48.2% of responses) (N = 236). About 25% reported that the vaccine was costly and they did not have the time to receive it. An additional 17.8% believed they were at low risk of HPV infection. Other reasons included inconvenient healthcare accessibility, fear of needles, and lack of knowledge about HPV. In the survey, 490 male youth who were not vaccinated against HPV were asked about their intention to get the vaccine in the future. Supplementary Table 3 (Table S3) Their responses showed that address region, sexual orientation, and prior receipt of information about HPV infection and vaccination were significantly associated with intention to get the vaccine. Most of the participants did not feel the need to get vaccinated, although more than 50% of Thai male youth from the South expressed their intention to get the vaccine, which was higher than any other region. In terms of sexual orientation, two-thirds of the heterosexual male youth were not willing to get the vaccine, while the proportion of homosexual and bisexual males willing to get the vaccine, 47.3% and 52.3%, respectively, was quite similar. Most participants who had heard about HPV but had not received the vaccine (58.3%) stated that they would not get vaccinated. However, the proportion of those who intended to receive the vaccine was higher in the group that had previously heard about HPV.

Previous receipt of information about HPV infection and vaccination

The aim of this study was to investigate the knowledge and awareness of HPV, the HPV vaccination rate, and reasons behind vaccination decisions of Thai male youth. The study found that previous receipt of information about HPV was a significant determinant in all aspects of the study. This suggests that providing accurate information is crucial for future clinical applications. Supplementary Table 4 (Table S4) displays the sources from which the participants learned about HPV. Social media was the most common source of information for Thai males, followed by healthcare providers who contributed more than half of the respondents' information. Two sources, friends and publications such as newspapers and magazines, both also had a strong effect in spreading information being cited in 42.1% and 35.7% of responses, respectively. Television, family, and other sources had only a minor influence.

DISCUSSION

This study, which included 594 participants, aimed to assess the knowledge and awareness of HPV and HPV vaccination among Thai male youth. In the study, the median score for knowledge was 11 (maximum score 18) (IQR 8-13). Several factors, including higher education level, higher family income, bisexuality, and prior receipt of information about HPV were associated with significantly higher knowledge of HPV scores. In terms of awareness (full scale 100%), the study found that the median awareness was 80% (IQR 68-92), and that previous receipt of HPV information was the only factor significantly associated with higher awareness. The study also found that the HPV vaccination rate among Thai male youth was 4.2%, and that most of the vaccinated individuals had decided to receive the vaccine by themselves. However, most participants who had not been vaccinated responded that they did not know that men could get HPV vaccination. Only 37.3% of the unvaccinated group expressed an intention to get vaccinated. Interestingly, unvaccinated males from the South region, those who were homosexual

or bisexual, and individuals who had previously received HPV information were significantly associated with a higher likelihood of getting vaccinated in the future. Participants indicated they had heard of HPV infection and vaccination mostly through social media, followed by healthcare providers, friends, and print media.

There was no difference observed in HPV knowledge between different age groups. However, recent evidence from Germany has shown that knowledge of HPV tends to increase with age (17). Some German schools now include sex education, including STD diseases, in their curriculum, which might have increased knowledge about HPV (17). In this study, male students pursuing high vocational certificates demonstrated significantly higher knowledge scores than those who completed only junior high school. This result suggests that the difference may be attributable to variations in the cultural norms of sexual education between academic and vocational education programs. We strongly advocate for sex education to be integrated into school curriculums, as there is a correlation between education level and higher knowledge scores.

Individuals with family incomes exceeding 50,000 baht per month tended to have a significantly higher score on knowledge of HPV. This finding could be attributable to the fact that higher income families have greater access to education and healthcare resources. Additionally, bisexual men displayed higher knowledge levels than heterosexual men, which is consistent with a study conducted among Australian women. In that study, bisexual women were found to have a greater awareness of HPV compared to heterosexual women (18). Individuals who identify as bisexual may be more knowledgeable and better prepared when it comes to engaging in sexual activities with partners of both genders. This could be due to their need to educate themselves on various sexual practices as well as on the risks of sexually transmitted infections and prevention strategies. As a result, they may have a better understanding of sexual health and safety practices than those who do not identify as bisexual.

Men who were better informed about HPV had higher scores for knowledge, awareness, and vaccination intention. This is because having prior knowledge about HPV infection and vaccination helps in understanding the potential risks and severity of diseases caused by this virus, its mode of transmission, and how to prevent it. In our study, over half of the men had heard of HPV (53.2%). This proportion is similar to a study among adolescent boys in England (19), but was considerably higher than in many countries where only 10-30% of people had heard of HPV (20-23). From the questionnaire, only a small number of participants indicated they had received information about HPV infection and vaccination in the classroom. Surprisingly, the majority of participants (70.6%) had learned about HPV and HPV vaccine from social media. This finding could have a significant impact on knowledge, awareness, and attitudes towards HPV infection and vaccination. It is crucial to note that helping individuals learn how easily HPV can be contracted can increase their awareness of the issue. According to our survey, only 4.2% of respondents reported that they had received the HPV vaccine. The exact number of HPV-vaccinated men in Thailand is unknown. Globally, only 4% of males have completed the entire course of the HPV vaccine (24). As shown in Table S5 of our study, there are two primary reasons why some participants did not receive the HPV vaccination. The first is lack of knowledge about HPV infection, including the misconceptions that men cannot receive the HPV vaccine and that there is only a low risk of getting infected, coupled with a lack of awareness of the vaccine. The second reason is the high cost of the vaccine, which is only provided free of charge for 11-12-year-old girls under Thailand's healthcare benefits system. Unfortunately, others must bear the cost of the vaccine themselves, which is a significant financial burden for many families, particularly amid the ongoing economic crisis. The survey found that 183 respondents (37.3%) intended to get vaccinated. This percentage is similar to the 41% vaccination intention rate found in England (19). Certain cultural beliefs and practices related to region or race have also been shown to affect awareness of HPV and the vaccine. In this study, participants residing in the Southern region were more likely to have received the HPV vaccine compared to other regions. A similar dichotomy was found in a study that reported Hispanics residing in the US were less aware of HPV and the HPV vaccine than non-Hispanic Whites. (25).

It's important to note that a person's sexual orientation can affect their decision to get vaccinated. Studies have shown that young gay men in the US and Thailand are more likely to consider getting vaccinated against HPV than are heterosexual men. This information can be useful for vaccination campaigns to help improve overall vaccination rates (26).

Strengths and limitations

The survey was conducted among Thai male youth from all major regions of the country using stratified random sampling, resulting in accurate and geographically inclusive data collection. The questionnaire system ensured that each email account was able to respond only once, thus avoiding repetitive answers. Additionally, the survey guaranteed respondents' confidentiality and anonymity, which encouraged them to provide truthful and realistic answers.

It is important to note that this study also has certain limitations. First, the survey did not include the levels of knowledge, awareness, and vaccination intention of female youth regarding HPV, which could have been used for comparison. The approach requiring internet access for participation in the questionnaire survey may have introduced potential selection bias, possibly contributing to the observation that nearly 40 percent of participants reside in the central region. Consequently, the results of this study may not be representative of the entire Thai male youth population. Additionally, the survey did not provide probable reasons to explain why education levels and geographic regions were associated with HPV knowledge and vaccination intention.

Clinical application and future direction

Based on the findings of this study, it appears that most of the participants lacked knowledge about HPV infection and vaccination, underlining the importance of raising awareness and encouraging young males to receive the vaccine and to better educate themselves about the disease. A previous study suggested that the HPV vaccine should be administered before the first sexual intercourse. Our data indicates that Thai men typically have their first sexual experience between the ages of 16 and 19. As a result, we recommend encouraging vaccination before that age. The Thai health education curriculum for school-aged children should include information on sexually transmitted infections and the importance of HPV vaccination. Social media can also be a valuable resource for promoting the HPV vaccine. Lastly, it is essential to gather more comprehensive, nationwide information on HPV vaccination among both males and females.

CONCLUSIONS

Although there is a high level of awareness of HPV infection and vaccination among Thai male youth, the current level of knowledge is still insufficient. Additionally, their acceptance rate of the HPV vaccine is still low and their attitudes are largely based on misconceptions. Therefore, it is important to develop improved education strategies, especially for this age group, to improve their understanding of HPV and increase their intention to get vaccinated. Such strategies could lead to a decrease in HPV-related diseases and help induce herd immunity in the future.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ADDITIONAL INFORMATION

Authors' contributions

All authors contributed to the study's conception and design. T.C., T.R.: performed material preparation and data collection. P.I.: reviewed Thai male population data. C.N., S.S., G.P.: analyzed and interpreted the patient data. C.N., S.S., G.P.: were major contributors in writing the manuscript. All authors read and approved the final manuscript.

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Table S1. Frequency distribution of knowledge of HPV and HPV vaccine knowledge (N = 594).

Questionaire items	Correct response	Yes (%)	No (%)	Unsure (%)
1. HPV can be transmitted to sexual partner regard-less male or female	Yes	462 (77.8)	22 (3.7)	110 (18.5)
2. Men can receive HPV vaccine	Yes	434 (73.1)	23 (3.9)	137 (23.1)
3. Even though receiving HPV vaccine, individuals still have risk of HPV infection	No	179 (30.1)	183 (30.8)	232 (39.1)
4. HPV vaccine is safe	Yes	361 (60.8)	41 (6.9)	192 (32.3)
5. HPV can cause herpes	No	215 (36.2)	153 (25.8)	226 (38.0)
6. Multiple sexual partners increase the risk of HPV infection	Yes	516 (86.9)	11 (1.9)	67 (11.3)
7. HPV infection increases the risk of penile cancer	Yes	376 (63.3)	50 (8.4)	168 (28.3)
8. HPV infection increases the risk of anorectal can-cer	Yes	275 (46.3)	81 (13.6)	238 (40.1)
9. HPV infection increases the risk of nasopharyn-geal cancer	Yes	188 (31.6)	118 (19.9)	288 (48.5)
10. Sexual intercourse at an early age increases risk of HPV infection	Yes	303 (51.0)	107 (18.0)	184 (31.0)
11. Using condom can reduces risk of the infection	Yes	532 (89.6)	9 (1.5)	53 (8.9)
12. Smoking is not a risk factor of HPV infection	No	91 (15.3)	330 (55.6)	173 (29.1)
13. For couples who received HPV vaccine, condom is not necessary	No	36 (6.1)	468 (78.8)	90 (15.2)
14. Sexual intercourse can increase risk of HPV in-fection, only if partner shows sign or symptom of HPV infection	No	108 (18.2)	355 (59.8)	131 (22.1)
15. HPV can be transmitted via contact or sharing common utensils	No	274 (46.1)	131 (22.1)	189 (31.8)
16. HPV vaccination in male youth requires 3 doses	Yes	224 (37.7)	56 (9.4)	314 (52.9)
17. Individuals who already have sexual intercourse have no necessity to get HPV vaccine because they were infected	No	32 (5.4)	408 (68.7)	154 (25.9)
18. Universal coverage scheme and social security fund cover the cost of HPV vaccination in Thai-land	No	224 (37.7)	85 (14.3)	285 (48.0)

Table S2. Frequency distribution of awareness of HPV knowledge and HPV vaccine (N = 594).

Questionnaire item	Strongly disagree (%)	Disagree (%)	Neutral (%)	Agree (%)	Strongly agree (%)	Mean	S.D.
1. You believe that you are risky to be HPV in-fected and should receive HPV vaccine	75 (12.6)	91 (15.3)	178 (30.0)	108 (18.2)	142 (23.9)	3.3	1.3
2. You believe that being HPV infected are really caused life threatening disease	6 (1.0)	21 (3.6)	65 (10.9)	215 (36.2)	287 (48.3)	4.3	0.9
3. All students regardless of their gender should be received HPV vaccine	5 (0.8)	5 (0.8)	90 (15.2)	166 (27.9)	328 (55.3)	4.4	0.8
4. You would suggest your friend to receive HPV vaccine	7 (1.2)	8 (1.3)	143 (24.1)	187 (31.5)	249 (41.9)	4.1	0.9
5. You believe that HPV vaccine is capable to prevent other type of HPV-infected cancer	13 (2.2)	32 (5.4)	166 (27.9)	171 (28.8)	212 (35.7)	3.9	1

Table S3. Association between characteristics of unvaccinated Thai male youth and intention to get HPV vaccine (N = 490, Chi-square test)

Variables	Total	N (% within a row)		P-value
		Yes	No	
1. Education				0.48
Junior High school	46	17 (37.0)	29 (63.0)	
High school	139	53 (38.1)	86 (61.9)	
University	238	94 (39.5)	144 (60.5)	
Vocational certificate	39	9 (23.1)	30 (76.9)	
High vocational certificate	27	10 (37.0)	17 (63.0)	
Non - formal Education	1	0 (0.0)	1 (100.0)	
2. Region				0.02*
Central	185	58 (31.4)	127 (68.6)	
North	91	35 (38.5)	56 (61.5)	
Northeast	152	58 (38.2)	94 (61.8)	
South	50	29 (58.0)	21 (42.0)	
East	10	2 (20.0)	8 (80.0)	
West	2	1 (50.0)	1 (50.0)	
3. Religion				0.52
Buddhism	443	161 (36.3)	282 (63.7)	
Christianity	10	5 (50.0)	5 (50.0)	
Islam	3	1 (33.3)	2 (66.7)	
Atheist	34	16 (47.1)	18 (52.9)	
4. Family income (bath per month)				0.13
Less than 25,000	90	29 (32.2)	61 (67.8)	
25,000-50,000	146	57 (39.0)	89 (61.0)	
50,001-75,000	67	27 (40.3)	40 (59.7)	
75,001-100,000	49	26 (53.1)	23 (46.9)	
More than 100,000	68	22 (32.4)	46 (67.6)	
5. History of sexual intercourse				0.26
No	314	111 (35.4)	203 (64.6)	
Yes	152	62 (40.8)	90 (59.2)	
6. Sexual orientation				< 0.01**
Heterosexual	338	110 (32.5)	228 (67.5)	
Homosexual	74	35 (47.3)	39 (52.7)	
Bisexual	44	23 (52.3)	21 (47.7)	
Others	6	4 (66.7)	2 (33.3)	
7. Previous receiving information about HPV infection and vaccination				0.03*
No	226	73 (32.3)	153 (67.7)	
Yes	264	110 (41.7)	154 (58.3)	
Total	490	183 (37.3)	307 (62.7)	

*; P < 0.05, **; P < 0.01, statistically significant

Table S4. Sources of HPV infection and vaccination information (N = 316)

Sources	N (%)
Social media	223 (70.6)
Healthcare provider	162 (51.3)
Friends	133 (42.1)
Print media (e.g., Newspaper, magazine etc.)	113 (35.7)
Television	71 (22.5)
Family	57 (18.0)
Others	8 (0.03)

Table S5. Frequency distribution of rationales contributing to the decision-making of HPV vaccination (N = 515).

	N (%)
Yes (N = 25, 4.9%)	
Self-decision	17 (68.0)
Parents	14 (56.0)
Healthcare workers	9 (36.0)
Friends	1 (4.0)
No (N = 490, 95.1%)	
Unknowing that men can receive HPV vaccine	236 (48.2)
HPV vaccine is costly	125 (25.5)
Not enough time to get HPV vaccination	121 (24.7)
Self-perception that individual has low risk to HPV infection	87 (17.8)
Difficulty in transportation	76 (15.5)
Trypanophobia	63 (12.9)
Unknowing about HPV infection and vaccination	20 (4.1)
No history of sexual intercourse	6 (1.2)
Others	9 (1.8)

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Exploring Synergies of Lotus Seed Extract-Hyaluronic Acid Gel for Enhanced Local Drug Delivery

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ABSTRACT

OBJECTIVE The plant species *Nelumbo nucifera* (lotus) is widely used in traditional medicine and is known to contain flavonoids, alkaloids, and other polyphenols which contribute to its potent antioxidant, anti-inflammatory properties in addition to its being a remedy for cardiac diseases. Hyaluronic acid, present in skin and connective tissue, is well known for its tissue regeneration and wound healing properties. The present study aimed to determine the antioxidant and anti-inflammatory effect of the hyaluronic acid gel combined with lotus seed extract and how this combination could be used as an effective local drug delivery system for the treatment of periodontitis.

METHODS A 2% solution of hyaluronic acid gel was combined with increasing concentrations of prepared lotus seed extract. A DPPH test was conducted to determine the antioxidant activity of the resultant mixture at increasing concentrations. Additionally, anti-inflammatory activity was assessed using a UV spectrometer, and the spreadability of the gel was measured using the sliding glass slide method. The values obtained were plotted on graphs.

RESULTS The DPPH scavenging assay revealed that lotus seed extract with 2% hyaluronic acid gel exhibited increased antioxidant activity in a dose-dependent manner with IC_{50} at 76 µL. The lotus seed extract with 2% hyaluronic acid gel also showed increased anti-inflammatory properties in a dose-dependent manner with IC_{50} at 271 µL. Moreover, the spreadability of the lotus seed extract with 2% hyaluronic acid gel was found to be 42 mm.

CONCLUSIONS Lotus seed extract shows potent antioxidant and anti-inflammatory activities which vary with the concentration of the extract, and can serve as an effective local drug delivery system.

KEYWORDS flavonoids, lotus seed, hyaluronic acid, local drug delivery, quality of life, periodontitis

INTRODUCTION

Periodontitis is one of the most commonly encountered inflammatory diseases affecting teeth and supporting structures. A mechanical therapy involving root surface debridement is an efficient way of treating periodontitis. However, that method does not reduce the presence of microbes in tissues or other areas inaccessible to instrumentation. It is essential to incorporate antimicrobial therapy to enhance the efficacy of traditional treatment methods. Antimicrobial therapy can be in the form of either systemic or local delivery modes.

Local drug delivery aims for specific site delivery without systemic side effects, as seen in systemic antimicrobial therapy. In this study, we synthesized an HA-lotus seed extract gel for local drug delivery.

Lotus seed extract, derived from the seeds of the sacred lotus plant (*Nelumbo nucifera*), is renowned for its traditional medicinal uses in various cultures. Flourishing in aquatic environments, this perennial plant is recognized for its exquisite flowers and distinctive pad-like leaves (1). Lotus seed extract is rich in phytochemicals, offering numerous health benefits (2). *Nelumbo nucifera* contains a variety of phytochemicals, including saponins, alkaloids, polyphenols and carbohydrates. Its potent antioxidant activity, derived from its composition which includes flavonoids and phenols, gallic acid and chlorogenic acid, plays a vital role in preventing oxidative stress. The total phenolic content of the lotus plant studied by Leong et al. was found to be the highest in the seeds, with a range of 20.6 to 38.3 mg TAE/g of extract. These compounds neutralize free radicals (act as scavengers), reducing the risk of chronic diseases (3). The leaves and stems of the lotus plant are well known for exhibiting anti-inflammatory properties by boosting the natural defense cells in the body, thereby reducing the inflammatory disease affecting the body (4). However, the anti-inflammatory and antioxidant property in the lotus seeds was found to be the most effective as analyzed by Rai et al. The radical scavenging activity, assessed by the IC_{50} values, was found to be 6.12 ± 0.41 µg/mL (2), hence the present study focused on the antioxidant activity and anti-inflammatory activity and their combination with hyaluronic acid (HA) in the treatment of periodontitis. These bioactive components are also believed to enhance cognitive function and promote mental well-being (5).

HA is a naturally occurring anionic glycosaminoglycan found in connective tissues, joints, and skin. The most striking feature of this acid is its water retention capacity. In gel form, it acts as a lubricating agent and provides exceptional hydration to the cells and tissues (6). For that reason, HA is incorporated in cosmetic products, the demand for which has increased rapidly. It also exhibits viscoelastic behavior, allowing it to absorb any disturbances and shocks, and providing a cushioning effect to the joints (7). In gel form, it creates a conducive environment for the migration of cells and regeneration of tissues. This gel is utilized in various medical applications, including wound dressings and post-surgery treatments (8). HA gel in combination with lotus seed extract is known to display anti-edematous, anti-bacterial and pro-angiogenic properties. Previous studies have shown that decreased levels of gingival inflammation and increased periodontal attachment are found when antioxidant status is improved with HA gel. As reported by Asieh et. al, the IC_{50} values of HA gel were found to be around 55 ± 0.7 mM and demonstrating its substantially high radical scavenging properties (9). Because the antioxidant activity of the lotus seed extract and HA gel was found to be significant, the synergistic effect of these compounds was chosen for use in local drug delivery systems for periodontitis.

Unlike conventional systemic drug delivery systems, which circulate medications throughout the entire body, local drug delivery is able to focus on application and injection of drugs into targeted sites. This effectively reduces the potential damage to surrounding tissues while at the same time diminishing any side effects (10). These systems employ advanced technologies for the meticulous administration of the drugs directly to the intended area through microneedles, implants, or patches (11). This precise targeting is advantageous for treating chronic pain and inflammation, as well as localized bacterial and viral infections. The single most desirable significant advantage of local drug delivery is its ability to achieve desired concentrations at the target site, thus enabling the maximum beneficial effect of the drug and, at the same time, minimizing exposure to the drug (12).

Lotus seed extract can be encapsulated within carriers like nanoparticles, liposomes, or hydrogels. This encapsulation facilitates controlled release, ensuring a sustained localized delivery of therapeutic agents. The present study assessed the antioxidant and anti-inflammatory effect of lotus seed extract in combination with HA and its potential for further enhancement as a local drug delivery agent in the treatment of periodontitis.

METHODS

Preparation of the lotus seed HA

Organic lotus seed was purchased from a local market in T. Nagar, Chennai, India. 50 g of the seeds was soaked in 500 ml of 95% ethanol. The solution was left undisturbed at room temperature for two days, after which a fine filtrate was

obtained by filtration through a Whatmann filter paper. A 2% HA gel was combined with increasing concentrations of the plant extract. Encapsulated lotus seed extract with HA gel was prepared as per the method described by Mohammad et al. (13).

Test for antioxidant activity

To 100 mL of ethanol, 4 mg of DPPH was added to obtain a concentration of 0.1 mM. The lotus seed extract was then added at increasing concentrations (25, 50, and 100 mg/mL) to the prepared DPPH solution. The radical scavenging activity was observed at 517 nanometers by measuring the intake of the different solutions. As a reference, pure DPPH solution (20 mg/mL) was tested for antioxidant activity at the same wavelength (14). The percentage of radical scavenging activity of the samples was calculated using the following formula:

$$\% \text{ RSA} = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100$$

where RSA is the radical scavenging activity, Abs control is the absorbance of DPPH radical + ethanol and Abs sample is the absorbance of sample.

For the study, gallic acid was chosen as the positive control due to its high antioxidant capacity. The antioxidant activity of the extract was compared with gallic acid, as it is known to have high free radical scavenging activity. The DPPH solution mixed with ethanol was used as a negative control to eliminate errors and miscalculations during incubation, as shown in Figure 1.

Test for anti-inflammatory activity

Different extract concentrations (25, 50, and 100 mg/mL) were added to 1.5 ml of bovine serum albumin (2% solution produced with 0.05 M Tris HCl). Tris HCl was used to alter the pH of the final solution. The samples were incubated for 30 minutes, then the prepared samples were submerged in water which was then heated to 75°C for ten minutes, after which the samples were brought to room temperature. The turbidity of the samples was measured using an ultraviolet spectrophotometer at 660 nanometers, and the percentage inhibition of albumin denaturation was calculated.

$$\text{Anti-inflammatory (\%)} = \frac{\text{Optical density}_{\text{control}} - \text{Optical density}_{\text{sample}}}{\text{Optical density}_{\text{control}}} \times 100$$

Test to assess spreadability

Two grams of the prepared gel was placed on a glass slide measuring 75 x 25 mm using a pipette. Then, a second glass slide, approximately 1 mm thick, was used to spread the gel which was then placed over the first glass slide. The time taken for the solution to spread evenly over the slide was calculated, and the spread diameter was measured after 1 minute.

RESULTS

Antioxidant activities of lotus seed extract with 2% hyaluronic acid gel

The values obtained from each test were tabulated and graphs were plotted. The antioxidant properties of lotus seed extract with 2% HA gel were determined by using the DPPH scavenging assay. The 1,000 µg/mL of gallic acid was used as a positive control and the antioxidant activity of the positive control was set as 100%. The results indicated that the lotus seed extract with 2%

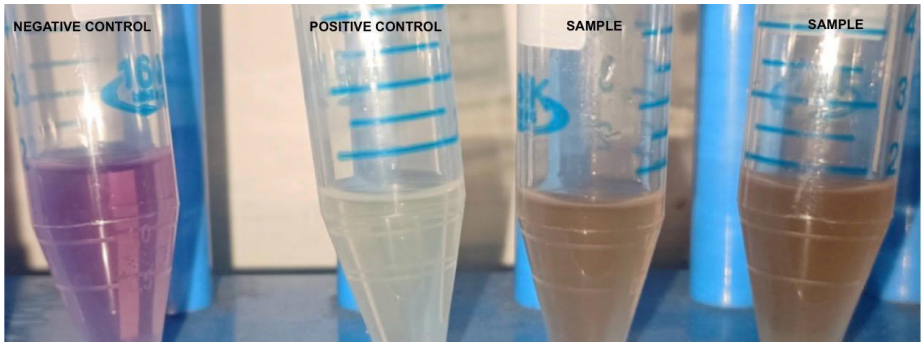

Figure 1. Samples containing ethanolic extract of lotus seed in combination with 2% HA gel. Positive control – Gallic acid (1,000 µg/mL); negative control – ethanol solution.

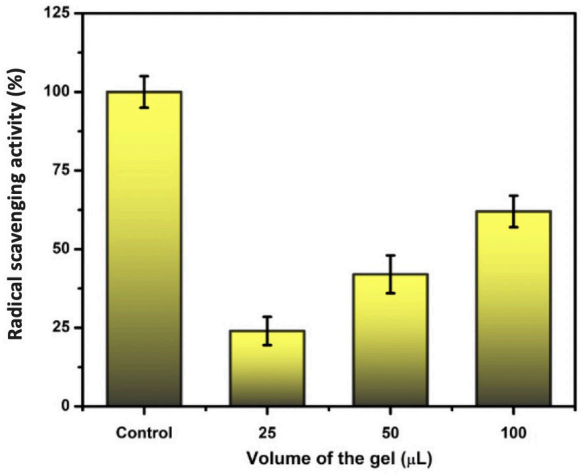

Figure 2. Graph depicting the antioxidant activity of ethanolic extract of lotus seed-HA at 25, 50, and 100 µL compared with the control (1,000 µg/mL of gallic acid).

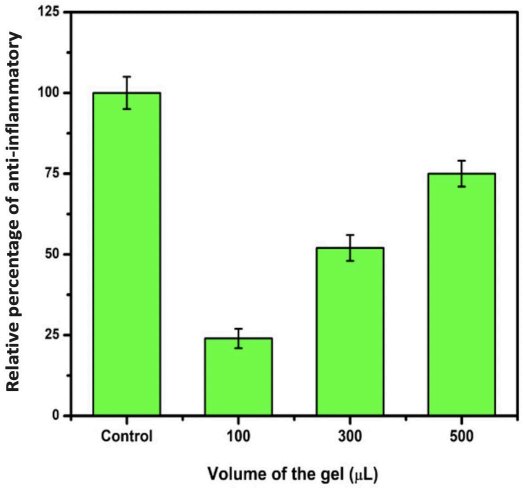

Figure 3. Graph depicting the anti-inflammatory activity of ethanolic extract of lotus seed-HA at 100, 300, and 500 µL compared with the control (1,000 µg/mL of gallic acid).

HA gel increased antioxidant activity in a dose-dependent manner. At 25, 50, and 100 µL of the lotus seed extract with 2% HA gel, the radical scavenging activity was found to be 25%, 37%, and 62%, respectively, as shown in Figure 2. The volume of the extract that was required to scavenge 50% of the initial DPPH radicals (IC_{50}) was shown to be 76 µL.

Anti-inflammatory activities of the lotus seed extract with 2% hyaluronic acid gel

The values obtained from each test were tabulated and graphs were plotted. The anti-inflammatory properties of lotus seed extract with 2% HA gel were determined using the BSA denaturation inhibition assay. The 1,000 µg/mL of gallic acid was used as a positive control and the antioxidant activity of the positive control was set as 100%. The results revealed that lotus seed extract combined with 2% HA gel exhibited increased anti-inflammatory activity in a dose-dependent manner. At 100, 300, and 500 µL of the lotus seed extract with 2% HA gel, the anti-inflammatory activity was found to be 25%, 61%, and 75%, respectively, as shown in Figure 3. Moreover, the volume of the extract that was required to inhibit 50% of the BSA denaturation (IC_{50}) was shown to be 271 µL.

The spreadability of the lotus seed extract with 2% hyaluronic acid gel

The spreadability of gel preparations refers to their ability to spread evenly across the skin's surface. The gel diameter was calculated by measuring the diameter of the gel on several sides. From this study, the spreadability of the lotus seed extract with 2% HA gel was found to be 42 mm, as shown in Figure 4.

DISCUSSION

The destruction of periodontal supporting tissues is due to the host immune inflammatory response triggered by red-complex bacteria (15). The present study evaluated the effectiveness of lotus seed- HA as a local drug delivery agent for the scavenging of free radicals as estimated by its antioxidant and anti-inflammatory ability (16). Ironically, in the present cosmetic era, use of natural remedies and herbal treatments has become increasingly popular for treating ailments such as cancer, skin diseases, poisoning, and periodontitis

Figure 4. Figure depicting the spreadability of the prepared extract (Lotus seed-HA)

(16, 17). The ethanolic extract of *Nelumbo nucifera* showed potent antioxidant and anti-inflammatory properties. Lotus seeds are the major reservoir and host to biologically active compounds such as flavonoids, tannins, and lignins (18). The hydroxyl groups in flavonoids alters their properties by chelating metal ions and breaking the antioxidant chain (19). Ascorbic acid, glutathione, and unsaturated fatty acids further enhance the antioxidant activity, making it a potent free radical scavenger (20). This activity is substantially amplified by the increased superoxide dismutase enzyme activity.

HA is widely used in periodontal therapy as adjunct to scaling and root planing and has resulted in greater reduction in probing depth and relative attachment level (21). It has also been used widely as nonsurgical treatment of papillary recession in esthetic areas. However, the use of HA injections provides only short term results (22). The use of HA gel as a drug delivery vehicle relies on its excellent biocompatibility and moisture-retaining capacity. These properties make it an ideal candidate for delivering therapeutic agents locally (23). Since HA is naturally present in the human body, the number of adverse allergic reactions and side effects to this acid are minimal (24).

The encapsulation of lotus seed extract within the HA gel ensures sustained and even release of the drug and prolongs the duration of the drug's interaction with tissues in the body. This property helps expand the duration of the effectiveness of the drug in addition to allowing concentration control (25). Additionally, combining lotus extract and HA can cause deeper drug penetration into the targeted tissues with immediate intake (26). As the antioxidant activity of the lotus seed extract and HA gel has been found to be significant, the synergistic effect of these compounds has resulted in their use in local drug delivery systems for periodontitis. Interestingly, the biological properties of lotus seed can also potentially promote wound healing and regeneration of tissues by amplifying the angiogenesis process. These properties make this substance an excellent option for local drug delivery (27, 28).

Previous research has shown that the physical properties of gels infused with HA can affect antioxidant activities more directly compared to those infused with HA (29). Additionally, the growth of *P. gingivalis* is known to be inhibited by high molecular weight hyaluronic acid. However, these properties fluctuate based on the acid concentration and the cell's metabolism (15). HA has a high binding affinity for CD44 cells which is expressed on the surface of leukocytes, found predominantly in individuals with periodontitis. The HA reduces leukocyte recruitment and there by decreases gingival inflammation. This research may pave the way for developing personalized drug delivery systems for inflammatory conditions. By altering the proportion of HA based on the type and purpose of the drug used, healthcare providers can potentially provide effective and efficient treatments to help resolve the specific ailments of each individual (16).

The synergy between lotus extract and HA provides a promising route for drug delivery by offering improved bioavailability, reduced side effects, and enhanced specific therapeutic outcomes. This approach could potentially revolutionize methods of delivering pharmaceuticals.

CONCLUSIONS

The present study effectively demonstrated the antioxidant and radical scavenging activity of *Nelumbo nucifera* (lotus seed) and how the addition of HA gel enhances these properties in a concentration-dependent manner. The present study provides scope for advanced research in this area, thus potentially enabling the formulation of other local drug delivery systems using lotus seed extract. Future studies should include both *in vivo* and *in vitro* studies to evaluate the efficacy of lotus seed extract-HA as a local drug delivery system in the treatment of chronic periodontitis.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

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