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Original Article

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Clinical profile and outcomes of asymptomatic *vs.* symptomatic travellers diagnosed with COVID-19: An observational study from a coastal town in South India

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SUMMARY Despite the high number of coronavirus disease-19 (COVID-19) cases from India, there are few reports from India describing the clinical epidemiology of COVID-19. This study aimed to describe the clinical/epidemiological characteristics and outcomes of asymptomatic *vs.* symptomatic COVID-19 patients. This was a retrospective chart review of all admitted patients with COVID-19 above 18 years with a history of travel within one month of the admission. The patients were categorized into asymptomatic and symptomatic. The symptomatic patients were further classified into mild, moderate and severe. The demographic profile, risk factors, clinical features, laboratory parameters, treatment details and outcome of all patients were recorded. The clinical and laboratory parameters were compared between symptomatic patients and asymptomatic patients. Of the 127 recruited patients, 75 were asymptomatic. Of the 52 symptomatic patients, 41 patients were classified as a mild illness. The mean age of the patients was 44.5 ± 15 years. A total of 73 patients had one or more risk factors. The male patients were more commonly found to be symptomatic compared to female patients. Neutrophil-lymphocyte ratio, C-reactive protein and lactate dehydrogenase were significantly elevated in symptomatic patients. A total of five individuals required supplemental oxygen therapy, and one of them required mechanical ventilation. All the patients had favourable outcomes. Asymptomatic and mild illness form a significant proportion of positive patients and have excellent outcomes without therapeutic interventions.

Keywords Pregnancy, household contact, COVID-19, Presymptomatic, asymptomatic, transmission, SARS-CoV-2

1. Introduction

Severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2), the causative agent of coronavirus disease-19 (COVID-19) is peculiar in terms of transmission dynamics and clinical manifestations despite its genetic relatedness to other coronaviruses (*1*). The virus multiplies in the upper respiratory tract, and the peak viral load is reached even before the symptom onset. As a result, asymptomatic individuals can also transmit. In those who are symptomatic, the manifestations range from mild to severe, requiring intensive care. Studies have shown that the proportion of asymptomatic and mild illness is significantly higher and a tiny proportion of individuals develop a severe disease *(2-4)*. Therefore, predictive scores based on comorbidities and laboratory parameters have been suggested to identify those individuals that require higher levels of care (*5*). The treatment for COVID-19 has also been continuously evolving with many of the proposed drugs failing to show benefits in randomized controlled trials (*6*).

Despite the high number of cases, there are relatively fewer reports from India describing the clinical epidemiology of COVID-19. Most of the reports are from tertiary care hospitals that often underestimate the actual proportion of asymptomatic/mild illness as only symptomatic individuals with more distressing symptoms are predominantly admitted in the hospital. Similar to other parts of the globe, the initial cases in

India were from the travellers. As per the regulations, testing and hospital-based treatment (irrespective of symptoms) were recommended for all travellers in the early part of the pandemic. It was hypothesized that the study of a cohort of travellers with COVID-19 would represent a distinct cohort. Therefore, this study aimed to describe the clinical-epidemiological characteristics and outcomes of hospitalized COVID-19 patients with travel history.

2. Materials and Methods

The study was a retrospective chart review conducted at Dr TMA Pai Hospital, Udupi (dedicated COVID-19 centre under public-private partnership) after taking permission from the Institute's Ethics Committee. The study was registered with the Clinical Trials Registry of India. The clinical case records of all admitted patients above 18 years with COVID-19 (based on real-time reverse-transcriptase polymerase chain reaction assay) between 1st of May, 2020 and 10th of July, 2020 were screened for eligibility. Those with a history of travel (international/national/state) within one month of the admission were included. After recruitment, the data was entered into a pre-defined case record form. The patients will be categorized into following categories: asymptomatic (No symptoms throughout the course), presymptomatic (No symptoms at the time of swab but developed symptoms later) and symptomatic (symptoms at the time of swab). The symptomatic patients were further categorized into mild, moderate and severe (Table 1). All patients were managed according to the institution protocol that was regularly updated. Routine investigations were done in all the patients - complete blood count (CBC), liver function tests (LFT), renal function tests (RFT), C-reactive protein (CRP) and lactate dehydrogenase (LDH). Ferritin and d-dimer were done on a case to case basis. Asymptomatic patients with mild disease were managed conservatively. They were not given any antivirals, steroids or immunomodulators. Those patients with moderate/severe disease were treated with supplemental oxygen therapy, hydroxychloroquine, low molecular weight heparin and steroids. Awake prone positioning was administered in all patients requiring oxygen. Those who did not respond to oxygen therapy were managed with non-invasive ventilation and mechanical ventilation. Two negative swabs were mandatory for discharge as per the state guidelines in the early part of the study period. The following details were recorded: the demographic profile, risk factors for severe disease (age > 60 years, hypertension, diabetes, chronic kidney disease, chronic lung disease, coronary artery disease, immunosuppression), clinical features, laboratory parameters, Chest X-Ray findings, treatment details and outcome. Based on an online web calculator, the patients were categorized into low (0.7% probability), medium (7.3% probability) and high risk (59.3% probability) of acquiring critical (mechanical ventilation/intensive care/death) illness (*5*).

Data analysis: continuous data were presented as either mean with standard deviation (SD) or median with interquartile range (IQR) depending on the data distribution. The frequency of categorical variables was expressed in numbers and percentage. The clinical and laboratory parameters were compared between symptomatic patients and asymptomatic patients. Chi-square test was used for categorical variables, and independent *t*-test was used for quantitative variables. A *p*-value of less than 0.05 was considered significant. All analyses were done using SPSSv26.

3. Results

Of the 127 patients, 75 were asymptomatic, and 52 were symptomatic (Table 1). The median duration of illness at admission in the 52 symptomatic patients was 4 (IQR 2.25-6.75) days. The demographic details are summarized in Table 2. The mean age of the patients was 44.5 (SD-15) years. The risk factors for severe disease are summarized in Table 3. A total of 73 patients had one or more risk factors for severity. The median duration from the day of swabbing to admission was 4 (IQR 2-7) days. The clinical features of symptomatic patients are summarized in Table 4. The median duration of illness from travel to admission was 9 (IQR 6-18) days. The mean pulse rate at presentation was 88.8 (SD-14.3) per minute. The mean respiratory rate at presentation was 18.6 (2.9) per minute. The mean systolic blood pressure (SBP) and diastolic blood pressure (DBP) at presentation were 126.9 (SD-18.5) millimetre (mm) of mercury (Hg) and 81.3 (SD-11.3) mm of Hg respectively. The mean saturation (SpO2) was 97.8 (1.8) %. A total of seven patients had Chest X-ray infiltrates (including two patients who were diagnosed with tuberculosis).

Table 1. Breakup of travellers admitted with COVID-19

Staging	Symptoms	Clinical signs of pneumonia	Oxygen Saturation (SpO_2) & Respiratory rate (RR) cut off	Number (Percentage)
Asymptomatic	No	No	-	75 (59%)
Mild	Yes	No	RR < 24/min	41 (36.2%)
Moderate	Yes	Yes	RR 24-30/min or SpO_2 90-94%	2 (1.6%)
Severe	Yes	Yes	RR > 30/min, SpO_2 < 90%	3 (2.4%)

Table 2. Demographic details of the patients

Demographic details	Frequency (n = 127)	Percentage
Month of Admission		
May	41	32.3%
June	73	57.5%
July (till the 10th of July)	13	10.2%
Travel		
International	12	9.5%
National	107	84.2%
State	8	6.3%
History of contact with a positive patient	11	8.7%
Age		
18-30 years	27	21.2%
31-40 years	34	26.8%
41-50 years	16	12.6%
51-60 years	27	21.2%
> 60 years	23	18.1%
Occupation		
Unemployed	9	7%
Home-maker	35	27.6%
Hotel employee	22	17.3%
Manual labour	6	4.7%
Health care worker	4	3.1%
Student	4	3.1%
Others	47	37%

The laboratory parameters of the admitted patients have been summarized in Table 5. A total of two patients had leucopenia, while five patients had leucocytosis. A total of seven patients had thrombocytopenia. Neutrophil-lymphocyte ratio (NLR) was more than 3 in 31 patients. CRP was elevated (> 5 mg/L) in a total of 46 patients. LDH was elevated (> 280 U/L) in a total of 46 patients. Bilirubin was elevated (> 1.2 mg/dL) in four patients. Aspartate transaminase (AST) (> 40 U/L) and alanine transaminase (ALT) (> 40 U/L) were elevated in 18 and 21 patients respectively. Alkaline phosphatase (ALP) (> 105 U/L) was elevated in 23 patients. Creatinine was elevated (> 1.2 mg/dL) in five patients. Ferritin was done in 76 patients, and the median ferritin value was 202 (95.4-424.5) nanogram per millilitre. D-dimer was done in 56 patients with a median value of 0.3 (0.2-0.48) mcg/mL. D-dimer (> 0.5 mcg/mL) was elevated in 13 patients.

On comparing the clinical and laboratory manifestations of symptomatic *vs.* asymptomatic patients, male patients were more commonly symptomatic than female patients (Table 6). Platelet count, CRP, LDH, AST and ALT were significantly higher in symptomatic

Table 3. Risk factors in patients diagnosed with COVID-19

Clinical details	Total number of patients (127)		Total symptomatic (52)		Moderate/Severe (5)
	Frequency	Percentage	Frequency	Percentage	
Number of risk-factors					
0	52	40.9%	24	46.1%	3
1	39	30.7%	14	26.9%	2
2	19	15%	7	1.9%	0
More than equal to 3	17	13.4%	7	1.9%	0
Risk factors					
Age > 60 years	23	18.1%	9	17.3%	1
Hypertension	40	31.5%	17	32.7%	1
Diabetes mellitus	39	30.7%	16	30.8%	1
Pregnancy	14	11%	2	3.8%	0
Coronary artery disease	6	4.7%	2	3.8%	0
Chronic lung disease	4	3.1%	4	7.7%	0
Human immunodeficiency virus infection	2	1.6%	0	0%	0

Table 4. Clinical features of patients with COVID-19

Clinical details	Frequency	Percentage of total (n = 127)	Percentage of symptomatic (n = 52)
Fever	19	15%	36.5%
Cough	30	23.6%	57.7%
Sore throat	9	7%	17.3%
Rhinitis	6	4.7%	11.5%
Dyspnoea	6	4.7%	11.5%
Headache	3	2.4%	5.8%
Myalgia	2	1.6%	3.8%
Loss of taste	3	2.4%	5.8%
Loss of smell	1	0.8%	1.9%
Chest discomfort	1	0.8%	1.9%
Tachycardia (> 100 per minute)	25	19.7%	48%
Bradycardia (< 60 per minute)	3	2.4%	5.8%
Tachypnoea (> 20 per minute)	31	2.4%	59.6%
Hypotension	0	0%	0%
Hypoxia (< 94%)	5	3.9%	9.6%

Table 5. Laboratory parameters of patients diagnosed with COVID-19

Laboratory parameters	Mean (Standard deviation)	Median (Inter Quartile range)	Reference range
Total leucocyte count (/mcL)	7,300 (2300)		4,000-11,000
Neutrophil-lymphocyte ratio		2.3 (1.7-3.8).	1-3
Platelet count at admission (lakhs/mcL)	2.98 (1.08)		1.5-4
C-reactive protein (mg/L)		3 (1-10.5)	0-5
Lactate dehydrogenase(U/L)	283.4 (106.4)		125-220
Bilirubin (mg/dL)		0.5 (0.3-0.6)	0.3-1.2
Aspartate transaminase (U/L)		23 (18-30.7)	0-40
Alanine transaminase (U/L)		22 (16-32)	0-40
Alkaline phosphatase (U/L)	83.6 (25)		35-105
Creatinine (mg/dL)	0.81 (0.23)		0.7-1.2

Table 6. Asymptomatic *vs.* symptomatic COVID-19

Parameters	Asymptomatic (n = 75)	Symptomatic (n = 52)	p-value
Sex			0.004
Male (n = 84), %	42 (50%)	42 (50%)	
Female (n = 43), %	33 (77%)	10 (23.2%)	
Number of comorbidities, n (%)			0.589
0 (n = 52), %	28 (53.8%)	24 (46.1%)	
1 (n = 39), %	25 (64.1%)	14 (35.9%)	
> 1 (n = 36), %	22 (61.1%)	14 (38.9%)	
Hypertension, n (%) (n = 40), %	23 (57.5%)	17 (42.5%)	0.809
Angiotensin receptor blocker (n = 15), %	9 (60%)	6 (40%)	0.937
Diabetes (n = 39), %	23 (59%)	16 (41%)	0.990
Pregnancy (n = 14), %	12 (85.7%)	2 (14.3%)	0.03
Age > 60 years (n = 23), %	14 (60.9%)	9 (39.1%)	0.84
Mean total leucocyte count in /mcL (Standard deviation)	7460 (2243)	6973 (2294)	0.877
Median neutrophil lymphocyte ratio (Inter-quartile range)	2.3 (1.7-3.6)	2.4 (1.7-3.8)	0.850
Mean platelet in lakhs/mcL (Standard deviation)	2.8 (1.6)	3.2 (1.2)	0.04
Median C-reactive protein in mg/L (Inter-quartile range)	2 (1-5)	7 (1.2-51.2)	0.001
Mean Lactate dehydrogenase in U/L (Standard deviation)	253 (67)	315 (138)	< 0.001
Median Aspartate transaminase in U/L (Inter-quartile range)	21 (16-25)	27.5 (20.2-44)	< 0.001
Median Alanine transaminase in U/L (Inter-quartile range)	18 (14-27)	28 (20.2-48.7)	0.001
Mean Alkaline phosphatase in U/L (Standard deviation)	82.8 (24)	83.9 (26.3)	0.813
Mean Bilirubin in mg/dL (Standard deviation)	0.5 (0.2)	0.6 (0.3)	0.109
Mean Creatinine in mg/dL (Standard deviation)	0.8 (0.2)	0.9 (0.2)	0.384

patients than asymptomatic patients (Table 6).

A total of five individuals required oxygen. The day of oxygen requirement from the day of onset of illness ranged from 6-10 days. Two of these patients developed acute respiratory distress syndrome (ARDS) in the disease course, and one of them required mechanical ventilation. The risk of developing critical illness according to the COVID GRAM calculator was as follows: low (n = 61), moderate (n = 63) and high risk (n = 3). Of the five patients who eventually required oxygen, three were categorized as high risk while the other two were classified as moderate risk (*5*).

A total of 15 hypertensive patients were on angiotensin receptor blockers. The number of patients who were given hydroxychloroquine (HCQ), low-molecular-weight heparin, steroids and antibiotics (ceftriaxone) was 5, 5, 2 and 5, respectively. All patients recovered eventually and were discharged. The mean duration of admission was 10.6 (SD-4.7) days. Repeat polymerase chain reaction assay (PCR) to demonstrate negativity was done in 64 patients, and the mean duration from the first positive swab to the first negative swab was 15.3 (SD-5.5) days. This period was significantly different between the asymptomatic and symptomatic group (16.2 *vs.* 13.6 days, p-value-0.04).

4. Discussion

Asymptomatic transmission of COVID-19 was initially described in household contacts of positive patients from China (*7-9*). The percentage of asymptomatic COVID-19 in positive patients from China ranged from 1.2-11% (*10-13*). However, the rate of individuals with asymptomatic COVID-19 depends highly on the testing strategies as asymptomatic individuals are unlikely to report to the hospital. In estimates derived after statistical modelling from individuals evacuated from China or individuals trapped in the Diamond Princess cruise, the proportion of asymptomatic cases ranged from 17.9% to 30.8% (Table 7) (*3,4*). In our cohort,

Table 7. Studies on transmission dynamics of SARS-CoV-2

S.N	Author Details	Type of study	Population and Sample size	Results
1	Bai *et al.*, China	Familial cluster		5 patients infected from one asymptomatic patient
2	Zhang *et al.*, China	Familial cluster		4 patients infected from one asymptomatic patient
3	Luo Y *et al.*, China	Familial cluster		4/5 household contacts of a symptomatic physician were positive but asymptomatic
4	NCPERE team, China	Retrospective review of records	72,314 patients (including positive and suspected)	1.2% asymptomatic
5	Wang *et al.*, China	Retrospective review of records	1,012 non-critically ill positive patients	3% asymptomatic
6	Dong *et al.*, China	Retrospective review of records	2,135 positive paediatric patients	4.4% asymptomatic
7	Zhu *et al.*, China	Meta-analysis	3,062 positive patients	11% asymptomatic
8	Nishiura *et al.*, Japan	Statistical modelling	565 evacuated individuals (9 symptomatic positives, 4 asymptomatic positives)	Asymptomatic proportion- 31%
9	Mizumoto *et al.*, Japan	Statistical modelling	634 positive in Diamond princess cruise	Asymptomatic proportion- 18%
10	Hu *et al.*, China	Case series	24 asymptomatic positive patients at presentation	21 % eventually developed symptoms (presymptomatic)
11	Meng *et al.*, China	Case series	58 asymptomatic positive patients at presentation	28% eventually developed symptoms (presymptomatic)
12	An *et al.*, China	Case series	25 asymptomatic patients at presentation	36% eventually developed symptoms (presymptomatic)
13	Zhou *et al.*, China	Case series	13 asymptomatic patients at presentation	23% eventually developed symptoms (presymptomatic)
14	Samsami *et al.*, China	Case series	8 asymptomatic patients at presentation	25% eventually developed symptoms (presymptomatic)
15	Kimball *et al.*, USA	Outbreak	76 exposed patients (23 positives)	56% asymptomatic at presentation (77% of which were presymptomatic)
16	Arons *et al.*, USA	Outbreak	76 exposed patients (48 positives)	56% asymptomatic at presentation (89 % of which were presymptomatic)

because all travellers were screened irrespective of the symptoms and were admitted irrespective of the severity, the percentage of asymptomatic individuals was higher (59%).

It is pertinent to distinguish individuals who remain asymptomatic throughout and individuals who are asymptomatic at presentation but develop symptoms later (presymptomatic). The proportion of presymptomatic individuals in patients who are asymptomatic at presentation varies from 21-89% (Table 7) (*14-20*). In our study, the number of presymptomatic individuals was only three. The ability of an asymptomatic or a presymptomatic individual to transmit infection was initially questioned. Still, it was found in an outbreak from a skilled nursing facility in the USA, that viral load in all three groups (symptomatic, presymptomatic and asymptomatic) were equally high. Of the 24 specimens collected from presymptomatic individuals, 17 specimens were also viable on culture. In another study of 2001 contacts of 157 symptomatic and 146 contacts of 30 asymptomatic cases, infection rates were 6% and 4% respectively (*21*). The difference in infectivity of symptomatic and asymptomatic cases was found to be statistically insignificant by the authors.

The spectrum of symptomatic patients with COVID-19 ranges from mild to severe. In a large report from China, the proportion of mild patients among all symptomatic patients was 81%. The risk factors for severe disease in patients with COVID-19 ranges from cardiovascular disease, diabetes mellitus, hypertension, chronic lung disease and immunosuppressive conditions. In a study, the mean number of comorbidities in patients who died was 2.7 (*25*). In another study, the mortality rate was significantly higher in those with comorbidities than those without comorbidities (*26*). Similar to our study, male sex was associated with more severe illness (*27,28*). Older age has also been associated with severe disease and mortality (*25,29*). Infection with respiratory viruses (influenza, SARS-CoV-1) has shown to affect pregnant women disproportionately in terms of increased severity and adverse outcomes (*30*). The data on the effect of this virus on maternal and fetal well-being is still evolving. The percentage of asymptomatic pregnant patients ranges from 23 to 33%, while the percentage of severe pregnant patients ranges from 5% to 14% (*31-34*). In our series, a total of 14 patients in various trimesters of pregnancy were identified. Except for two pregnant patients who had a mild illness, all the other patients were asymptomatic (86%). In a case-control study by Li *et al.*, maternal complications were higher in pregnant women with suspected/confirmed COVID-19 compared to controls (pregnant women without COVID-19) (Table 8) (*35*). Like our study, high NLR, thrombocytopenia, transaminitis, raised

Table 8. Maternal and foetal outcomes of COVID-19 cases

S.n	Author Details	Type of study	Population and Sample size	Results
1	Breslin *et al.*, USA	Case series	43 COVID-19 positive pregnant patients	33% asymptomatic at presentation. 9% severe and 5% critical
2	Ferrazzi *et al.*, Italy	Case series	42 COVID-19 positive pregnant patients	37% required oxygen support (*21*)
3	Yang *et al.*	Review of published cases	114 COVID-19 positive pregnant women	5% of the patients had severe/critical illness
4	Yan *et al.*, China	Case series	116 COVID-19 positive pregnant patients	23% were asymptomatic at presentation, while 7% had severe symptoms
5	Li *et al.*, China	Case-control study	Cases- Pregnant women with COVID-19, Controls- Pregnant patients without COVID-19	Maternal complications were higher in cases

inflammatory marker, raised troponin and raised d-dimer are associated with poorer outcomes (*36-38*).

The most common initial symptoms in patients with COVID are fever, cough, myalgia, rhinitis, diarrhoea, loss of smell and taste (*26*). It is pertinent to note that even though several guidelines include fever as the entry criteria for suspicion, it is present only in 31-46% of the patients in various studies. Similar results were noted in our research as well (*26,39*). The smell and taste alterations in COVID are more subjective than objective, thereby explaining the wide range of prevalence (5 to 98%) in various studies (*40,41*). Most patients with mild symptoms at onset recover without any further progression. A fraction of these patients may develop dyspnea. Like our study, the mean duration of dyspnea development ranges from 5-8 days (*42,43*).

Compared to other studies that report the case-fatality in the range of 1-12%, our outcome was excellent despite our restricted use of antivirals (*22-24*). This could have been because of many reasons. Those patients who were relatively healthy would have decided to travel, and therefore, our cohort may have been healthier at baseline than other hospital cohorts. Since this was the beginning of the pandemic, all patients were tested and were admitted in an institutional setting. This would have led to the inclusion of more asymptomatic patients leading to early identification of worsening and prompt management. Also, because it was the early part of the pandemic, the hospital resources (human resources and beds) were adequate to manage these patients in the best possible manner.

Apart from the limitations associated with the study's retrospective nature, the discharge criteria changed in the middle of the study period. Consequently, time to negativity could not be calculated in all the patients.

The possibility of transmission from asymptomatic cases which form a significant proportion of total positive patients but are missed on symptom-based screening calls for adherence to preventive measures such as physical distancing, frequent handwashing, wearing of masks in the community and universal masking. Since patients with asymptomatic and mild illness have excellent outcomes without any therapeutic interventions, unnecessary and unproven medications should be avoided in such patients. These patients can also be managed in-home isolation (with monitoring) to decrease the burden on tertiary care hospitals.

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Original Article

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A digital scheme of human trials for the evaluation of functional foods

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SUMMARY In this study, we designed a method for conducting a human study by the following process. (1) The host computer stores the subject information. (2) The sample preparer prepares a food sample. (3) The subject (healthy human volunteer) sends the information of an intake of the food sample to the host computer, which creates an event entry for the event. (4) The medical professional (typically a physician) collects and stores the subject's blood sample in a container with the subject's identification (*e.g.*, ID number). (5) The sample analyst analyzes the blood biochemical profiles. (6) The host computer stores the blood biochemical data, and by matching the blood biochemical data with the subject IDs, a final analysis report will automatically be created. In this study, we also run a test case, based on this design, where we obtained a blood biochemical dataset from healthy volunteers. This scheme can reduce the cost of human trials for functional foods and will help acquiring the scientific basis of functional foods.

Keywords human trial, personal devices, functional food, natural food

1. Introduction

As we enter a super-aging society, people's interest in natural foods is increasing. To obtain scientific evidence on the health effects of natural foods, results using laboratory animals are considered. We have proposed invertebrate models to replace mammalian models, and have applied them to a range of research topics such as infectious diseases (*1,2*), immune activation (*1,3-5*), antibiotic discovery (*6,7*), and diabetes (*8-11*). However, the health effects of natural foods must ultimately be verified in humans, which poses challenges due to its cost ($3000-5000 per subject (*12*)) and ethical issues. In conventional human trials to investigate the effects of natural foods on humans, all elements of the trial should be managed by physicians, and ensuring cost effectiveness has been a major issue (*13*). Therefore, in this study, we attempted to propose a method for conducting human trials with the use of personal computing devices, with the aim of facilitating the practical application process of newly developed natural foods and promoting the use of natural foods that may contribute to public health.

In this study, we focused on the fact that, among the processes of human trials for natural foods, only the processes related to invasive medical procedures such as blood sampling needs a direct supervision by physicians, but the other processes do not necessarily need to be performed by physicians. On the other hand, when non-physicians conduct the invasive steps in the human test method for natural foods, it may potentially be difficult to ensure and monitor the intake of test samples by subjects. For this potential hurdle, we thought it possible to overcome it by registering the subject information and trial checkpoints in advance in a computer and setting up a reminder to each event, so that each subject will be tracked throughout the trial *via* a personal computer, smartphone, tablet, *etc.* This method can also visualize the progress and results of the trial to the administrator and will reduce the barriers to the implementation of human trials for natural foods, accelerating the development and use of natural foods based on scientific evidence.

2. Materials and Methods

2.1. Lactic acid bacteria strains and peroral intake

The lactic acid bacteria (LAB) strains used in this study

were *Leuconostoc carnosum* #7-2 (*14*) (LAB strain A) and *Lactococcus lactis* 11/19-B1 (*15*) (LAB strain B). Live cells of each strain were prepared at a dose of 10^{10} cells/day/subject and administered orally to the subjects for 27 days. Three healthy adult volunteers were recruited by Genome Pharmaceuticals Institute Co., Ltd. (Tokyo, Japan).

2.2. Measurement of Natural Killer (NK) Cell Activity

Blood samples were collected by a physician for each subject three times: one day before the start of sample intake (day -1), in the middle of the sample intake period (day 14), and one day after the end of sample intake (day 28). The collected blood samples were refrigerated and transported to the analyst on the day of collection, and the analyst measured the peripheral blood NK cell activity. Specifically, peripheral blood leukocytes (E) and ^{51}Cr-labeled K562 cells (T) were mixed and cultured at E/T = 20 for 3.5 hours, and the %lysis value was determined. All three subjects ingested the samples once a day for 27 days (81 out of 81 times (100%)). Regarding the time of ingestion, 70 out of 81 times (86.4%) were between post-breakfast and 10:30 a.m. ingestion, which was the time period set by the administrator. In addition, a situation occurred twice where one of the subjects took the sample later than the originally scheduled time.

3. Results

3.1. A scheme for conducting a human study using personal devices

As shown in Figure 1, the host computer stores the subject information so that the administrator can identify the subject in the trial. In the process, the sample preparer first prepares the sample for the subject (for per-oral administration) and fills the sample container. Then, the subject sends the information at the time of intake to the host computer, and the host computer creates an event entry at the time of intake. Next, a medical staff (physician) collects the subject's blood and stores it in a blood storage container (*e.g.*, a

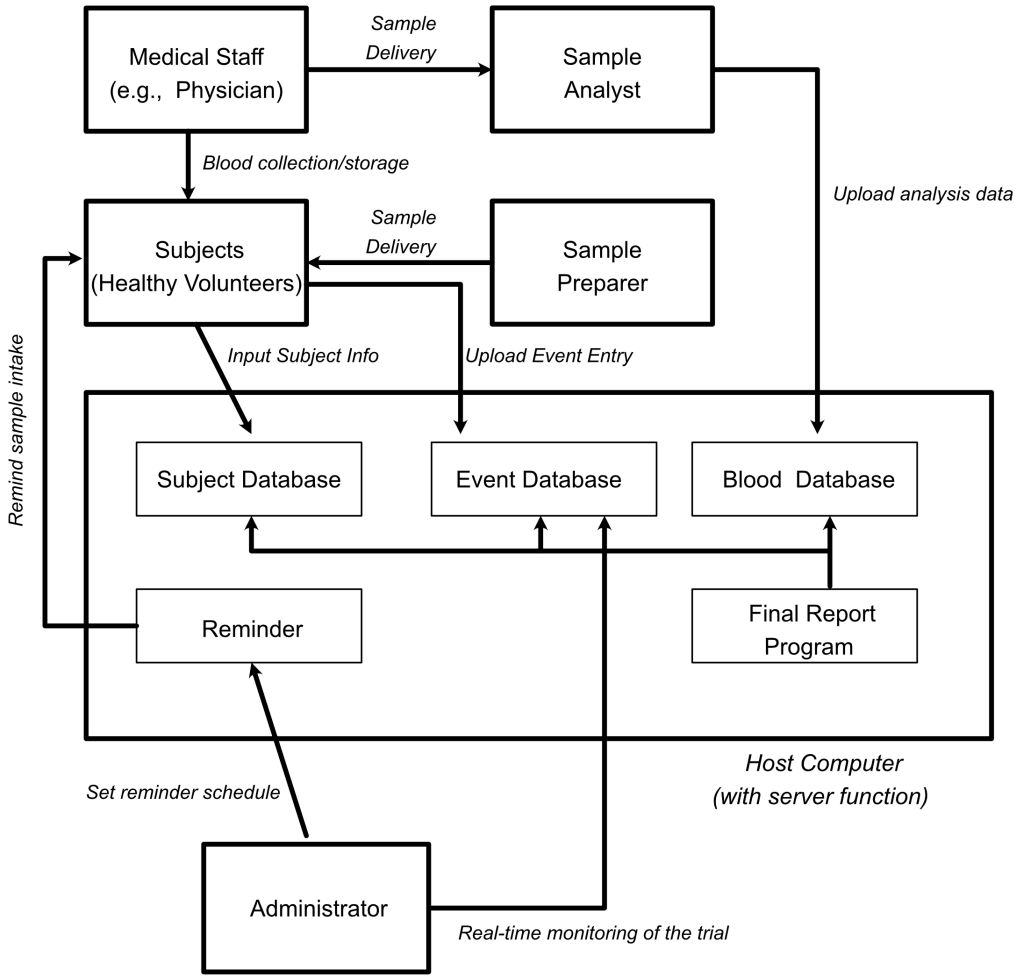

Figure 1. Processing scheme from data acquisition to analysis. This is a schematic diagram of the human trial method proposed in this study. Each of the thick rectangles represents each personnel or the host computer. The objects within the host computer represent a program, function, or storage devices.

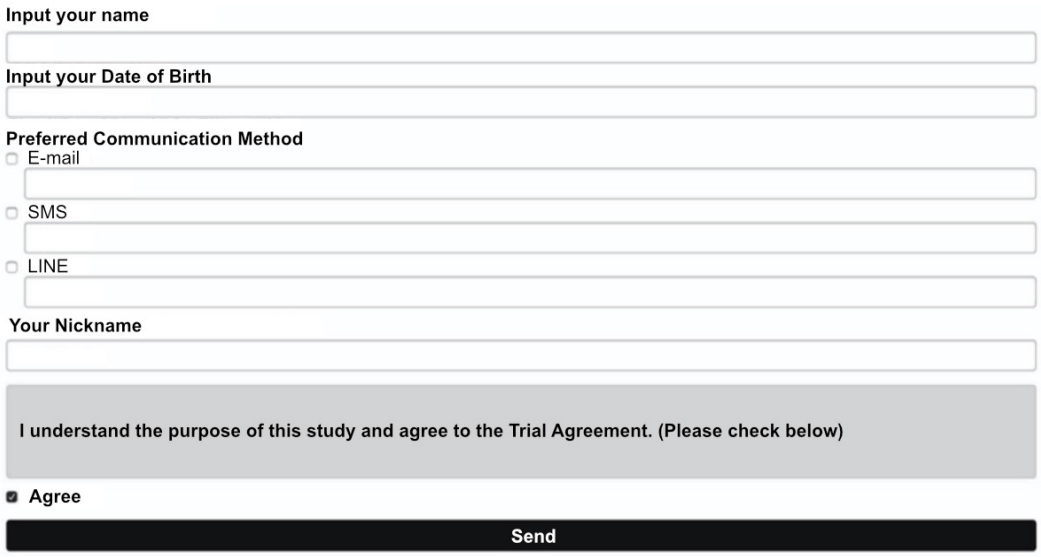

Figure 2. Example of input screen to the host computer. This is an example of the screen when the subject enters the subject data into the host computer.

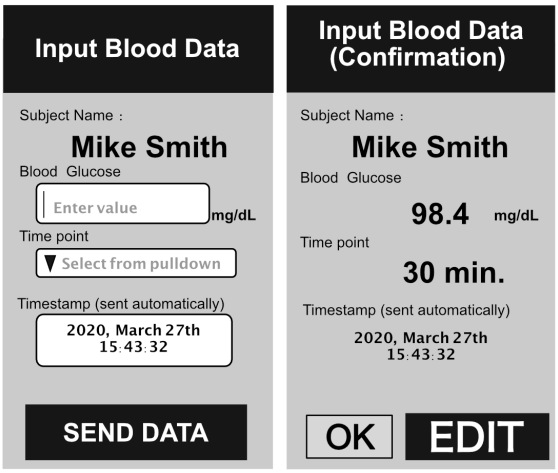

Figure 3. An example screen of subject-host computer communication. When a subject sends intake information (or the blood data in the case shown in the panel) to the host computer, the input screen (left) is displayed first, and then the data will be registered after the confirmation screen for data transmission (right).

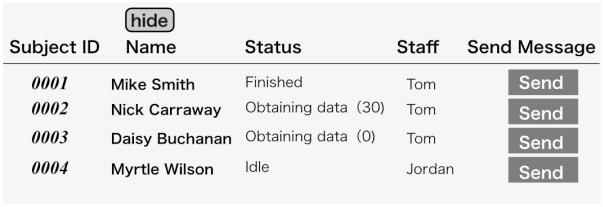

Figure 4. Example of the administrator screen. The administrator collectively manages the scheduled, in-progress, and completed studies. In the example of the operation screen shown in the figure, the entire study can be supervised in real time through the management screen where each subject can be listed.

sterile centrifuge tube) with the subject's identification. In addition, the sample analyst analyzes the blood and obtains the blood (*e.g.*, biochemistry traits or cell counts) analysis data including the corresponding identity of the subject (typically anonymized). Finally, the host computer stores the blood analysis data, and by matching the blood analysis data with the subject data and the intake (event) data, the host computer creates and stores the final report for the effect of the food sample. Figure 1 shows the entire scheme where the host computer integrates the whole processes of a trial.

In the case of actual operation, the subjects (*i.e.*, healthy volunteers) may first input their data by themselves into the host computer (an example input screen is shown in Figure 2). Then, once the blood data is obtained, each subject will connect to the web server from his or her personal terminal (smartphone, tablet, *etc.*) and upload the measurement data (examples input and confirmation screens are shown in Figure 3). Throughout the study, the administrator should supervise the execution of the study in real time through an integrated manager (an example window is shown in Figure 4).

3.2. A model case

As an example of human trial that the above-mentioned scheme can be used, a study to examine the effect of lactic acid bacteria on human blood Natural Killer cell activity is shown in this paper (Table 1). This experiment involved an administrator (a non-physician faculty of our university), three subjects (three healthy volunteers), a sample preparer (a researcher at our university), a medical worker (a physician faculty member), and an analyst (a staff from a company that owns analytical equipment) were involved in the study.

Table 1. Examples of human studies with repeated intake of lactic acid bacteria

Subject #	NK activity		
	day -1	day 14	day 28
Replicate 1 (LAB strain A)			
1	34	37	31
2	47	36	35
3	57	59	51
mean	46	44	39
SD	12	13	11
Replicate 2 (LAB strain B)			
1	41	39	37
2	23	30	17
3	51	63	40
mean	38	44	31
SD	14	17	13

NK cell activity is shown in the table. SD: standard deviation. Results from three healthy volunteers are shown in the table. See Materials and Methods section for details.

The three subjects orally ingested the sample once a day for 27 consecutive days. The samples were stored in the laboratory freezer within building where the trial was performed, and the sample delivery from the sample preparer to the subjects was smooth.

4. Discussion

In the human trial scheme proposed in this study, the administrator can easily monitor the progress of the trial in real-time. In addition, by developing and using appropriate software, the results of the trial (final report) can be provided in a visualized form (tables, graphs, *etc.*), which greatly reduces the labor required to publish the results of the human trial. This is important in promoting the participation of physicians and others in human trials for natural foods. This scheme can be implemented in a medical university with faculty members with or without medical license as administrators. In this case, it will be easy to recruit staff members, researchers, students, *etc.* at the university (who understand the significance of the trial and are likely to comply with the instructions of the administrator, thus ensuring the reliability of the test results) as volunteer subjects. Furthermore, the preparation of food samples can be done in university laboratories, so that the delivery of it to the subjects will be smooth as demonstrated in this study. This is especially important when handling food samples that may have potential stability issues.

Part of the human trial scheme is carried out by a host computer with server functions. This scheme requires knowledge of the programs and databases used to perform human trials, but both are standard skillsets in today's technology. Furthermore, the scheme can be applied to any human study in which the blood of a subject is collected to directly evaluate and analyze the health effects of food. For example, natural foods, health foods, functional foods, foods for specified health uses, and dietary supplements can be used. The blood collection from subjects is an invasive medical procedure, and the involvement of medical personnel such as physicians is essential in conducting the study. However, for processes other than blood collection, non-physicians can serve as administrators (*i.e.*, the person in charge of conducting the study, who oversees the communication among the parties involved in the human trial, data management, and compilation of results), thereby reducing the burden on researchers in conducting such studies. In conventional human trials, blood collection is usually conducted in hospitals, and medical personnel such as physicians serve as managers, imposing a heavy burden on the trials. In this scheme, non-physicians can act as administrators, and as a result, academia-derived human trials can be conducted at low cost. This scheme is expected to be widely used for human trials of natural foods, health foods, functional foods, food for specified health uses, dietary supplements, *etc.*, and it will enable reliable, smooth, and cost-efficient human trials.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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Original Article

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The safety of ritodrine hydrochloride: Adverse effects on fetuses and newborns

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SUMMARY Prematurely born infants face unique risks, and the treatment of imminent preterm birth is thus an important part of perinatal care. Ritodrine hydrochloride (Rito) is widely used as a therapeutic agent to treat imminent preterm birth in Japan. Following assessment of the risks and benefits of short-acting β-agonists, including Rito, in Europe, however, the use of Rito has begun to be questioned. Thus, in this study we investigated the safety of Rito in the treatment of imminent preterm birth, with a particular focus on the adverse effects (AEs) on fetuses and newborn infants. Using the Pharmaceuticals and Medical Devices Agency of Japan's Japanese Adverse Drug Event Report (JADER) database, the AEs on fetuses and newborns caused by oral and injected Rito were extracted and analyzed. The reported odds ratios for oral Rito were significantly higher for fetal tachycardia, fetal bradycardia, neonatal hypoglycemia, and neonatal heart failure than for other drugs. The reported odds ratios for Rito injection were significantly higher for fetal tachycardia and neonatal hypoglycemia than for other drugs. Oral drugs had more adverse effect reports than injectable drugs.

Keywords Ritodrine hydrochloride, pregnant women, fetuses, newborns, adverse effects

1. Introduction

Perinatal care strives to protect the health of fetuses and newborn infants. In recent years, the average age of first marriage for women has increased, and late marriage is becoming more frequent. With the increase in late marriage, the average maternal age is also on the rise (*1*). The Japan Society of Obstetrics and Gynecology (JSOG) defines advanced maternal age as primiparas more than 35 years of age. Advanced maternal age can increase the risk of preterm labor as a result of aged ova and increased chromosomal abnormalities of the fertilized egg. The JSOG defines imminent preterm birth as when regular uterine contractions are observed between 22 and 36 weeks of gestation and progress in the degree of cervical dilatation/extension is observed, or when the cervical canal is examined at the first visit and is dilated by 2 cm or more (*2*). Infants born prematurely face a variety of risks and challenges. Preterm birth in late pregnancy (34-36 weeks gestation) can result in respiratory problems, hypothermia, hypoglycemia, *etc.* Preterm birth at an earlier stage (less than 34 weeks gestation) poses risks such as severe respiratory distress, cerebral hemorrhage, and severe infections (*3,4*). Therefore, the treatment of preterm labor in perinatal medicine is important.

Rito and magnesium sulfate are covered by insurance in Japan as tocolytic agents used in the treatment of imminent preterm birth, and Rito is widely used (*5,6*). Drug selection and dose adjustment generally take into consideration the subjective symptoms of uterine contraction and length of the cervical canal. Rito has a selective β2 receptor-stimulating effect and is used in pregnant women after 16 weeks of gestation. Depending on the patient's symptoms, continuous infusion for 24 h by oral administration or infusion is performed. Although it has been reported that the administration of Rito prolongs the gestation period and increases the rate of term delivery (*7,8*), its use has begun to be questioned (*9,10*).

Currently, the use of Rito to treat preterm labor varies greatly from country to country. In October 2013, a risk/benefit assessment of short-acting β-agonists, including Rito, was conducted in Europe. For oral preparations, the cardiovascular risk was judged to outweigh the efficacy and approval was revoked (*11*). The use of injections was limited to a maximum of 48 h between the 22nd and 37th week of gestation due to concerns related to cardiovascular risk (*12*). At that time, there were few research reports recommending against long-term administration in Japan. Since long-term administration for the purpose of continuing pregnancy is a widely used treatment method, administration with careful monitoring for adverse

effects (AEs) has been considered a viable option.

Recently, however, there have been reports that question the efficacy of Rito and query if it should be withheld due to concerns related to serious AEs (*9,10*). Although pregnant women may need this medication, information related to its administration during pregnancy remains unclear and there is little information in the package insert. Against this background, there are many concerns about the effects on fetuses and newborns. Therefore, in this study, we analyzed the AEs on fetuses and newborns to determine the safety of Rito in the treatment of imminent preterm birth.

2. Methods

2.1. Usage survey of Rito usage

Using the Ministry of Health, Labour and Welfare's NDB Open Data, the amount of Rito (oral medicine and injectable) used from April 2014 to March 2018 was investigated.

2.2. Extraction of AEs on fetuses and neonates caused by Rito (oral medicine and injectable)

Using the Pharmaceuticals and Medical Devices Agency of Japan's Japanese Adverse Drug Event Report (JADER) database, we analyzed the adverse effect data reported for approximately 15 years (April 2004-December 2019), and extracted the AEs caused by Rito. Then, AEs related to fetuses and newborn infants counted. Further, the number of adverse effect reports for drugs other than Rito was counted for the AEs related to fetuses and newborn infants listed in Rito.

2.3. Analysis of AEs reporting frequency

To understand the frequency of AEs related to fetuses and newborn infants caused by Rito, a 2 × 2 contingency table was created for each adverse effect according to the presence or absence of Rito administration and the presence or absence of AEs. Then, the reported odds ratio (ROR) and the 95% confidence interval were calculated. For drugs other than Rito, the keywords "fetus," "newborn," "pregnancy," and "caesarean section" were used, and only cases in which it was clear that the patient was pregnant were extracted.

2.4. Comparison of AEs tendency between oral and injectable Rito

To compare the frequency of reports of AEs common to oral Rito and injectable drugs, a 2 × 2 contingency table was prepared according to administration method (oral administration or injection) and presence or absence of AEs. The ROR and *p*-value were calculated. The lnROR and -logP values were also graphed, and the frequency of reported adverse reactions with oral and injectable drugs was compared and examined.

2.5. Statistical analysis

Fisher's exact test was used to compare the two groups, and the level of significance was set at $p < 0.05$.

3. Results

3.1. Oral Rito

3.1.1. Usage status of oral Rito

Figure 1 shows the usage status of oral Rito by year and age. The amount of oral Rito used in FY2014 was smaller than in other years, but it was almost the same in FY2015-2017. In addition, when compared by age, the usage was highest in patients 30 to 34 years old, followed by 25 to 29 years old and 35 to 39 years old in all years. Although the amount used was smaller than that of the above age groups, it was also used in patients in their 40s and older, early 20s, and 15 to 19 years old.

3.1.2. Reporting frequency of AEs on fetuses and newborns

The total number of reported adverse reactions caused by oral Rito was 260. Adverse reaction in fetuses was 17, and in newborns was 33.

In fetuses, the most frequently reported adverse drug reaction was fetal tachycardia (5 cases, 29.4%). This was followed by fetal bradycardia (4 cases, 23.5%), fetal disorder (2 cases, 11.8%), and fetal death (2 cases, 11.8%). In addition, in order to examine the frequency of the reported AEs of Rito, we compared it with other drugs. As a result, the RORs for fetal tachycardia and fetal bradycardia were 9.468 (2.984-30.042) and 3.292 (1.093-9.920), respectively. The frequency of reported adverse reactions to Rito was significantly higher than

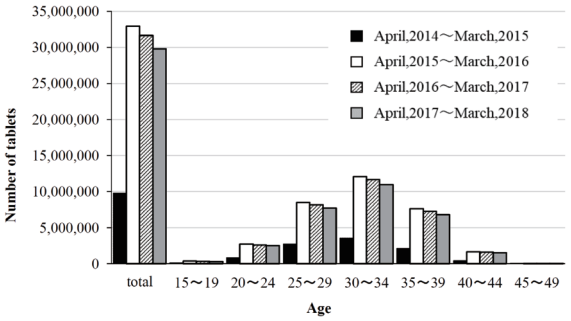

Figure 1. Annual usage of ritodrine hydrochloride (5 mg tablet). Using the Ministry of Health, Labour and Welfare's NDB Open Data, the amount of ritodrine hydrochloride (oral medicine) used from April 2014 to March 2018 was investigated.

Table 1. Adverse effects and reported odds ratios for oral ritodrine hydrochloride: Fetuses

Adverse effects	Number of reports		ROR (95% CI)	*p* value
	Rito	Other drugs		
	Total: 260	Total: 3387		
Fetal tachycardia	5	7	9.468 (2.984-30.042)	0.0000
Fetal bradycardia	4	16	3.292 (1.093-9.920)	0.0249
Fetal disorder	2	17	1.537 (0.353-6.688)	0.5639
Fetal death	2	91	0.281 (0.069-1.146)	0.0587

The data shows the top four adverse effects. Rito: ritodrine hydrochloride, ROR: reported odds ratio, CI: confidence interval.

Table 2. Adverse effects and reported odds ratios for oral ritodrine hydrochloride: Newborns

Adverse effects	Number of reports		ROR (95% CI)	*p* value
	Rito	Other drugs		
	Total: 260	Total: 3387		
Neonatal hypoglycemia	8	31	3.437 (1.563-7.555)	0.0011
Low birth weight infants	8	320	0.304 (0.149-0.621)	0.0005
Neonatal heart failure	5	10	6.622 (2.246-19.519)	0.0000
Neonatal asphyxia	3	187	0.200 (0.063-0.629)	0.0023

The data shows the top four adverse effects. Rito: ritodrine hydrochloride, ROR: reported odds ratio, CI: confidence interval.

that of other drugs. The RORs for fetal disorder and fetal death were 1.537 and 0.281, respectively, but no significant difference was observed (Table 1).

The most frequently reported adverse drug reaction in newborns was neonatal hypoglycemia (8 cases, 24.2%), followed by low birth weight infant (8 cases, 24.2%), neonatal heart failure (5 cases, 15.2%), and neonatal asphyxia (3 cases, 9.1%). As a result of comparison with other drugs, the ROR of neonatal hypoglycemia was 3.437 (1.563-7.555), and the ROR of neonatal heart failure was 6.622 (2.246-19.519). This result indicates that the frequency of reported adverse reactions due to Rito is significantly higher than that of other drugs. In contrast, the ROR for low birth weight infants was 0.304 (0.149-0.621) and the ROR for neonatal asphyxia was 0.200 (0.063-0.629). The frequency of these AEs due to Rito was significantly lower than that of other drugs (Table 2).

3.2. Rito injection

3.2.1. Usage status of Rito injection

Figure 2 shows the amount of Rito injections by year and age. The total amount of Rito injections was approximately the same in the four years from April 2014 to March 2018. By age, the usage was highest in patients 30 to 34 years old, followed by approximately the same usage in patients 25 to 29 years old and 35 to 39 years old. Although the amount used was smaller than that of the above age groups, it was also used in patients in their 40s and older, early 20s, and 15 to 19 years old.

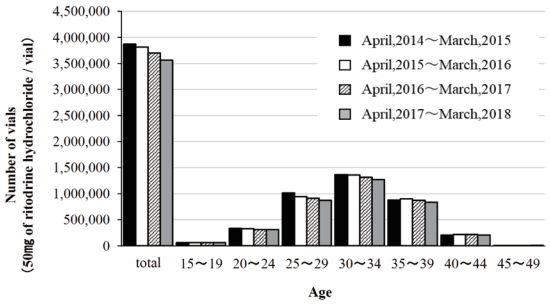

Figure 2. Annual usage of ritodrine hydrochloride injection. Using the Ministry of Health, Labour and Welfare's NDB Open Data, the amount of ritodrine hydrochloride (injectable) used from April 2014 to March 2018 was investigated.

3.2.2. Reporting frequency of AEs on fetuses and newborns

The total number of reported adverse reactions caused by Rito injection was 1281. Adverse reaction in fetuses was 48, and in newborns was 94.

The most frequently reported adverse reactions in fetuses were fetal tachycardia (10 cases, 20.8%), followed by fetal death (8 cases, 16.7%), transient fetal bradycardia abnormality (6 cases, 12.5%), and fetal bradycardia (5 cases, 10.4%). In comparison with other drugs, fetal tachycardia was significantly higher (ROR 3.556 [0.976-12.951]). In the following three items, the reported adverse effect rate of Rito was significantly lower than that of other drugs: fetal death (ROR 0.149 [0.071-0.314]), fetal transient bradycardia abnormality

Table 3. Adverse effects and reported odds ratios for ritodrine hydrochloride injection: Fetuses

Adverse effects	Number of reports		ROR (95% CI)	*p* value
	Rito	Other drugs		
	Total: 1281	Total: 1359		
Fetal tachycardia	10	3	3.556 (0.976-12.951)	0.0400
Fetal death	8	55	0.149 (0.071-0.314)	0.0000
Fetal transient bradycardia abnormalities	6	39	0.159 (0.067-0.378)	0.0000
Fetal bradycardia	5	44	0.117 (0.046-0.296)	0.0000

The data shows the top four adverse effects. Rito: ritodrine hydrochloride, ROR: reported odds ratio, CI: confidence interval.

Table 4. Adverse effects and reported odds ratios for ritodrine hydrochloride injection: Newborns

Adverse effects	Number of reports		ROR (95% CI)	*p* value
	Rito	Other drugs		
	Total: 1281	Total: 1359		
Neonatal hypoglycemia	23	21	1.165 (0.642-2.115)	0.6157
Low birth weight infant	21	86	0.247 (0.152-0.400)	0.0000
Preterm infant	9	87	0.103 (0.052-0.206)	0.0000
Neonatal asphyxia	8	126	0.061 (0.030-0.126)	0.0000

The data shows the top four adverse effects. Rito: ritodrine hydrochloride, ROR: reported odds ratio, CI: confidence interval.

(ROR 0.159 [0.067-0.378]), and fetal bradycardia (ROR 0.117 [0.046-0.296]) (Table 3).

In newborns, the most frequently reported adverse drug reaction was neonatal hypoglycemia (23 cases, 24.5%); this was followed by low birth weight infant (21 cases, 22.3%), preterm infant (9 cases, 9.6%), and neonatal asphyxia (8 cases, 8.5%). As a result of comparison with other drugs, the reported adverse effect rate of Rito for low birth weight infant, preterm infant, and neonatal asphyxia was significantly lower than that of other drugs (Table 4).

3.3. Comparison of adverse effect tendency between oral and injectable Rito

AEs common to oral and injectable drugs include fetal tachycardia, fetal bradycardia, fetal death, neonatal hypoglycemia, low birth weight infant, and neonatal asphyxia. The relationship between lnROR and -logP reveals that AEs tended to occur more easily with oral medication for all six reported adverse drug reactions (Figure 3). The order of frequency of occurrence was fetal bradycardia, fetal tachycardia, low birth weight infant, neonatal asphyxia, neonatal hypoglycemia, and fetal death. For fetal bradycardia, the frequency of AEs was significantly higher.

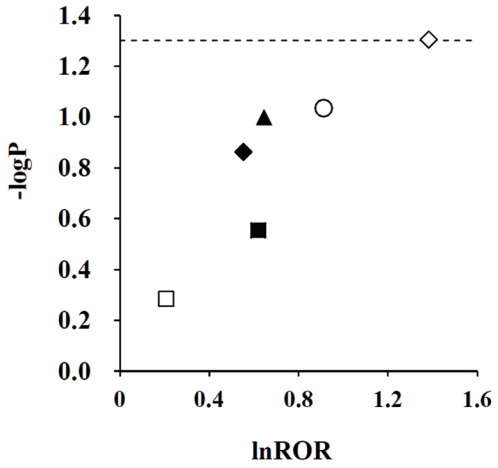

Figure 3. Comparison of the frequency of adverse effects common to oral ritodrine hydrochloride and injectable drugs. Fetal tachycardia (○), Fetal bradycardia (◊), Fetal death (□), Neonatal hypoglycemia (♦), Low birth weight infants (▲), Neonatal asphyxia (■), dotted line: -log0.05. lnROR and -logP values were graphed, and the frequency of reported adverse reactions with oral and injectable drugs was compared and examined. The positive direction on the horizontal axis tends to be more likely to occur due to oral administration, and the positive direction on the vertical axis indicates that there is a statistically significant difference.

4. Discussion

A survey of Rito usage revealed low usage in 2014. This was likely influenced by the October 2013 report on the domestic response to the restriction on the use of short-acting β-agonists, including Rito, in Europe. In all of the years surveyed, both oral and injectable drugs were used most frequently in patients between 30 and 34 years old, followed by those aged 25 to 29 and 35 to 39 years old. Although the amount used was smaller than in that

of the above age groups, Rito was also used in patients in their 40s and older, early 20s, and 15 to 19 years old. According to the data from the "Annual transition of the number of births by mother's age (5-year-old class) and birth order (2015-2018)" by the Ministry of Health, Labour and Welfare, the number of births was the highest at 30 to 34 years old, followed by 25 to 29 years old and 35 to 39 years old, indicating that usage was linked to birth status. Rito was also used by patients in their 40s and older. This may be due to the fact that as maternal age increases as a result of later marriage, and as the risk of imminent premature birth increases with age, the use of this medication becomes increasingly necessary.

Against this background, we analyzed the status of AEs reported in fetuses and newborns caused by oral and injectable Rito. We found that the RORs of oral Rito were significantly higher for fetal tachycardia, fetal bradycardia, neonatal hypoglycemia, and neonatal heart failure than for other drugs. The RORs for Rito injection were significantly higher for fetal tachycardia and neonatal hypoglycemia than for other drugs. They were therefore considered to be AEs unique to Rito. These adverse effect reports were consistent with the already reported package inserts (*12,13*).

Rito is more selective for uterine muscle than other β-agonists, such as isoxsuprine hydrochloride and isoproterenol hydrochloride. Many AEs, including fetal tachycardia and fetal bradycardia, have been reported due to the pharmacological action of stimulating β-receptors, however. Rito's longer period of action can lead to the postnatal effect of neonatal heart failure in infants. In addition, Rito has hepatic glycogenolysis and insulin secretion suppression effects, which are related to the increased maternal blood glucose effect. It is known that the rebound can cause transient hypoglycemia in postnatal infants (*14,15*). Hyperglycemia and diabetic ketoacidosis have been reported as serious adverse reactions to Rito in the mother. The onset of neonatal hypoglycemia can be suppressed by controlling the blood glucose at an early stage of the onset of maternal AEs.

The reporting frequency of the common AEs, such as fetal tachycardia, fetal bradycardia, fetal death, neonatal hypoglycemia, low birth weight infant, and neonatal asphyxia, between oral and injectable Rito was compared. All six AEs tended to occur more easily with oral medicine than with injectable drugs. The treatment of imminent preterm birth in Japan may be related to the long-term use of oral medication. Although it is not possible to directly compare the number of people in the usage survey, it is presumed that there is a large difference in usage and that oral medications are often used. In addition, reports from pharmaceutical companies also indicate that oral medications are often used in Japan.

As for the domestic administration status of Rito preparations (April 2012-March 2013), of the 2688 treatment groups, 2148 (79.9%) were treated with Rito alone, and 113 (4.2%) were treated with intravenous drip alone. Both oral and infusion treatments were reported in 427 patients (15.9%) (*11*). According to the pharmaceutical Interview Form, the number of AEs per daily average dose increased as the doses of both oral and injectable drugs increased (oral medicine: 11-15 mg [2.2%], 16 mg [4.1%]; injectable medicine: 151-200 μg [17.5%], 201 μg [31%]) (*16,17*). It is known that the number of adverse drug reactions for each period of use is highest 3 days after the start of administration (oral medicine 1.3%; injection 14.0%), however, and does not change significantly, even if the period of use is extended (*12,13*).

All six reported adverse drug reactions in fetuses and newborn infants investigated herein were found to be more likely to occur with oral drugs than with injectable drugs. In fetuses and newborns, long-term administration of this drug may increase the risk of AEs compared to short-term administration. In Europe and the United States, short-term administration within 48 h by injectable drug is recommended. The results herein indicate that the treatment period should be as short as possible.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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Dry fabrication of poly(*dl*-lactide-co-glycolide) microspheres incorporating a medium molecular drug by a ball mill method

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SUMMARY Poly(*dl*-lactide-co-glycolide) acid (PLGA) microspheres is a useful carrier for controlled drug release. However, the organic solvents used in their conventional manufacturing process may affect the chemical structure of a macromolecular drug. Thus, we investigated the applicability of a dry fabrication method for PLGA microspheres. Cyanocobalamin (MW = 1,355) (VB_{12}) was used as a model drug, and it formed agglomerates under mild conditions with powdered PLGA in a generic ball milling system. Light and scanning electron microscopy showed the formation of PLGA microspheres and few agglomerates. The obtained microspheres had the particle size injectable as suspensions, namely smaller than 150 μm specified for subcutaneous and intramuscular injections by the Japanese Pharmacopoeia. The observed and theoretical drug contents were consistent. PLGA microspheres fabricated using a combination of small (ϕ3 mm) and large (ϕ10 mm) balls showed low initial burst of cyanocobalamin release *in vitro*. The *in vitro* drug release profile was equivalent with that of the microspheres fabricated by a conventional oil-in-water emulsion solvent evaporation method, while the drug release profile was influenced by the brand of the PLGA used. To prevent drug loss during fabrication, the dry fabrication method using a ball mill should be applied to prepare PLGA microspheres containing a medium macromolecular drug.

Keywords biodegradable polymer, microspheres, solvent-free, long-acting injectable formulation, mechanical alloying

1. Introduction

Biodegradable polymeric microspheres are injectable formulations that promote long-acting drug efficacy in clinical practice. Although oral formulations are widely used for patient care, these formulations still hold some risks, such as risks of noncompliance, asphyxiation due to deterioration of swallowing ability, cognitive decline, polypharmacy, and finite supervised administration. Given these risks, there is a growing importance of long-acting injectable formulations administered under supervision.

Conventional methods for fabricating biodegradable polymeric microspheres include the emulsion-based solvent evaporation method (*1*) and the spray drying method (*2*). However, most biodegradable polymers used in long-acting injectable formulations, such as polylactide (PLA) and poly(*dl*-lactic-co-glycolic) acid (PLGA), are insoluble in an aqueous solvent. Thus, in the preparation of microspheres using the conventional methods, organic solvents, such as methylene chloride, are essential to dissolve the polymers. However, the use of organic solvents in the fabrication of microspheres leads to problems that can increase product cost, such as installation of a removal facility dedicated for volatile organic compounds, risk control for exposure of the environment and workers to organic solvents, and residual solvents in the formulation. Following the recent launch of biomedicines as therapeutic agents for refractory or rare diseases, a paradigm shift of therapeutic agents from chemical compounds to biomedicines is expected in drug development. However, the use of organic solvents in the manufacturing process of biomedicines can inactivate them, thus limiting the development of long-acting injectable formulations of biomedicines.

In the fabrication of biodegradable polymeric microspheres, not only the use of organic solvents but also the complicated manufacturing process leads to high production costs. The manufacturing processes of microspheres are, so far, more complicated than those of the conventional injectable formulation. The complicated processes are a disadvantage in terms of the aseptic guarantee because these processes are performed under aseptic conditions. In addition, containment technology is also needed if the

active pharmaceutical ingredients encapsulated in microspheres are highly potent.

We have investigated and proposed a new microsphere fabrication method without the use of halogenated solvents (*3*). Although the method results in low toxicity in the manufacturing process, the risks mentioned above remain. To completely solve these problems, a new fabrication method without the use of any solvents is needed. Recently, we reported a dry coating method (*4*). The method is based on the agglomeration that occurs in pulverization using a ball mill. Dry grinding with a ball mill is a popular technique used in the pharmaceutical industry; moreover, low critical particle sizes can be achieved through pulverization. In general, it is difficult to reduce particle size below 10 μm by dry milling. Core particles of approximately 10 μm are not pulverized in the dry milling process; however, the exothermic effect is maintained. Exothermic energy can melt the coating materials, leading to the formation of agglomerates of core particles and coating materials. The remarkable characteristics of this method is that coating materials larger than core particles can be applied. The dry coating technology used in the present study is related to mechanofusion, which is mainly used for modifying the surface of particles. For the conventional mechanofusion and dry coating procedures, smaller particles were used as guest particles and larger particles as hosts (*5-8*). In this case, guest particles corresponded to the coating materials. Nanoparticles are often used as guest particles to obtain particles of micrometer size, which refers to nanocomposites (*9,10*). However, there are some technical challenges in the fabrication of small guest particles, such as nanoparticles. Our reported technique has an advantage of no technical difficulties in obtaining coating materials.

The objective of this study is to evaluate the feasibility of our dry coating technique in the fabrication of PLGA microspheres. We compared the microspheres fabricated by the dry technique with those prepared by the conventional oil-in-water (o/w) solvent evaporation technique in terms of encapsulation efficiency and drug release profiles. The results of this study will encourage the use of an alternative, facile method for fabricating long-acting injectable formulations, such as PLGA microspheres.

2. Materials and Methods

2.1. Materials

PLGA with a lactic acid: glycolic acid ratio of 50:50 and inherent viscosity midpoint of 0.2 dl/g was purchased from Sigma-Aldrich Co., LCC. (St. Louise, US; Resomer® RG502 and Resomer® RG502H) and FUJIFILM Wako Chemicals Corporation (Osaka, Japan; PLGA5020), and was kindly gifted by Corbion (Amsterdam, Netherlands; PURASORB®PDLG5002). Cyanocobalamin (VB_{12}) was purchased from Enzo Life Science, Inc. (New York, US). Polyvinyl alcohol (PVA, POVAL® 220C) was obtained from KURARAY Co., Ltd. (Tokyo, Japan). All other chemicals used were of reagent grade. There are no ethical aspects to declare in this study.

2.2. Fabrication of microspheres

A. Dry fabrication method

Briefly, 150 mg mixture of pulverized VB_{12} particles (9.07 ± 0.47μm) and PLGA (21.86 ± 0.45μm) was placed in an agate ball mill pot (ϕ40 mm; h 40 mm) with balls (agate ϕ10 mm and zirconia ϕ3 mm). The ball mill was then rotated at 22-23°C using a planetary mill (Pulverisette 6; FRITSCH GmbH, Idar-Oberstein, Germany). One cycle consisted of rotation at a speed of 250 rpm for 30 min followed by resting for 5 min. The fabrication condition of each sample is shown in Table 1.

B. Conventional o/w emulsion solvent evaporation method

For microsphere fabrication using the conventional o/w emulsion solvent evaporation method, 30 mg pulverized VB_{12} particles (ca. 10 μm) were dispersed, and 120 mg PLGA were dissolved in 0.75 mL methylene chloride to prepare the oil phase. The oil phase was emulsified into 0.5% PVA aqueous solution 5 mL using a homogenizer (ULTRA-TURRAX® T18; IKA®-Werke GmbH & Co., KG, Staufen, Germany) for 5 min at 22-23°C. The resultant emulsion was added to 100 mL water all at once as needed and stirred at room temperature to remove the solvent for 3 h. This constituted the solvent evaporation process. The hardened particles were sieved through 149-μm sieves to remove any aggregates and collected by 20-μm sieves to be lyophilized.

2.3. Microscopic observations

The obtained microspheres were observed by a scanning electron microscopy (SEM) (JSM-5500LV; JOEL Ltd., Tokyo, Japan). Samples for SEM observation were prepared through deposition on a gold-palladium plate at 15 mA for 3 min (Quick Auto Coater JFC-1500; JOEL Ltd.).

2.4. Determination of particle size

The sizes of the fabricated microspheres were determined using a laser diffraction particle size analyzer (SALD-2200; Shimadzu Co., Ltd., Kyoto, Japan). Briefly, A few mg the microspheres were dispersed in 0.05% tween 80 solution and the suspension were applied to the analyzer.

Table 1. Fabrication condition of VB_{12} microspheres

Sample No.	Fabrication Method	VB_{12}/PLGA (VB_{12} loading)	PLGA	Ball[2)]	Cycle[3)]	Total process time
#1	Dry fabrication method	30 mg/120 mg (20%)	Resomer®RG502	4 agate balls	2	1h
#2	Dry fabrication method	30 mg/120 mg (20%)	Resomer®RG502	4 agate balls	6	3h
#3	Dry fabrication method	30 mg/120 mg (20%)	Resomer®RG502	4 agate balls	12	6h
#4	Dry fabrication method	30 mg/120 mg (20%)	Resomer®RG502	4 agate balls	24	12h
#5	Dry fabrication method	15 mg/135 mg (10%)	Resomer®RG502	4 agate and 4 zirconia balls	24	12h
#6	Dry fabrication method	15 mg/135 mg (10%)	Resomer®RG502	4 agate and 10 zirconia balls	24	12h
#7	Dry fabrication method	15 mg/135 mg (10%)	Resomer®RG502	4 agate and 15 zirconia balls	24	12h
#8	Dry fabrication method	15 mg/135 mg (10%)	Resomer®RG502	4 agate balls	24	12h
#9	Dry fabrication method	30 mg/120 mg (20%)	Resomer®RG502	4 agate and 15 zirconia balls	24	12h
#10	Dry fabrication method	45 mg/105 mg (30%)	Resomer®RG502	4 agate and 15 zirconia balls	24	12h
#11	Conventional method[1)]	30 mg/120 mg (20%)	Resomer®RG502	-	-	-
#12	Dry fabrication method	30 mg/135 mg (20%)	PURASORB®PDLG5002	4 agate and 15 zirconia balls	24	12h
#13	Dry fabrication method	30 mg/135 mg (20%)	Resomer®RG502H	4 agate and 15 zirconia balls	24	12h
#14	Dry fabrication method	30 mg/135 mg (20%)	PLGA5020	4 agate and 15 zirconia balls	24	12h

[1)] o/w emulsion solvent evaporation method, [2)] agate ball: ϕ 10 mm, zirconia ball: ϕ 3 mm, [3)] milling for 30 min followed by resting for 5 min.

2.5. Determination of VB_{12} content in microspheres

Microspheres loaded with VB_{12} (10 mg) were weighed in a test tube and dissolved in methylene chloride (2 mL), to which 9.6 mM phosphate buffered saline (pH 7.4; 5 mL) was added. VB_{12} was extracted after overnight shaking. After centrifugation for 5 min at 2,000 rpm, the upper aqueous layer was collected, and the concentration of VB_{12} in the aqueous layer was determined by the absorbance at 360 nm using a hybrid multimode microplate reader (Synergy H4; BioTek Instruments, Winooski, LV, USA) based on absorptiometry. VB_{12} loading and encapsulation efficiency were calculated using the following equations:

$$VB_{12}\ \text{loading}\ (\%) = \frac{\text{Weighed } VB_{12}\ (\text{mg})}{\text{Weighed } VB_{12}\ (\text{mg}) + \text{Weighed PLGA (mg)}} \times 100$$

$$\text{Encapsulation efficiency}\ (\%) = \frac{\%\ \text{actual } VB_{12} \text{ content in microspheres}}{\%\ VB_{12} \text{ loading in microspheres}} \times 100$$

2.6. Dissolution test

Dissolution test was performed by the rotating bottle method. Briefly, microspheres loaded with VB_{12} (10 mg) were weighed in a test tube, to which 9.6 mM phosphate buffered saline (pH 7.4; 10 mL) at 37°C was added as a dissolution medium. The microspheres were then dispersed, rotated at 25 rpm at 37°C in an air-conditioned incubator (BioShaker V-BR-36; TITEC, Koshigaya, Japan), and collected at predetermined times. After centrifugation for 1 min at 2,500 rpm, all the medium was collected from the tube as a sample for dissolution assay and replaced with a fresh medium (10 mL). The dissolution test was performed in triplicate. The VB_{12} that remained after the dissolution test was extracted and determined using the method described above.

2.7. Related substance analysis by HPLC

The aqueous layer obtained in the 'Determination of VB_{12} content in microspheres' section was further analyzed by high-pressure liquid chromatography (HPLC) (Prominence; SALD-2200; Shimadzu Co., Ltd.) under the conditions of purity test in the Japanese Pharmacopoeia 17 edition. Namely, VB_{12} and the related substances in the aqueous layer were assayed under the following conditions: detector: an ultraviolet absorption photometer (wavelength: 361 nm); column: a stainless steel column of 4.6-mm inside diameter and 25-cm length, packed with octylsilanised silica gel for liquid chromatography (5-µm particle diameter) (InertSustainC8, GLsciences, Tokyo, Japan); column temperature: a constant temperature of approximately 30°C; mobile phase: A (70 mM Na_2HPO_4 adjusted to pH 3.5 with phosphoric acid) and B (methanol) at an A/B ratio of 53/147; and flow rate: 1.05 mL/min.

2.8. Fourier transform infrared ray spectrometry (FT-IR)

Microspheres were analyzed by an attenuated total reflection method by FT-IR (FT/IR6600; JASCO International Co., Ltd., Tokyo, Japan). The samples were placed on a φ2.5-mm diamond prism and scanned at a range of 500-4,000 cm^{-1}.

2.9. Statistical analysis

Experiments were replicated at least thrice. The results are presented as means ± standard error of the means (s.e.m.).

3. Results

3.1. Fabrication conditions

First, we investigated the effect of processing time at

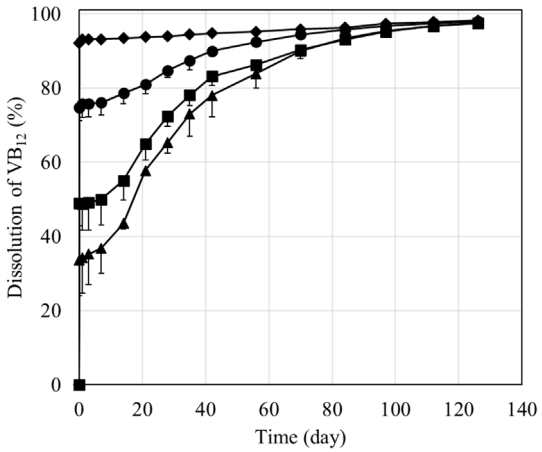

Figure 1. Effect of processing time on *in vitro* VB_{12} release from the microspheres fabricated by the dry fabrication method. The processing time was ♦: 1 h (sample No.: #1); ●: 3 h (sample No.: #2); ■: 6 h (sample No.: #3); ▲: 12 h (sample No.: #4). The microspheres were fabricated under the following conditions: media, four balls (ϕ10 mm); PLGA, Resomer®RG502; loaded drug, 20%VB_{12}. The data represent mean ± S.E. (n = 3 batches).

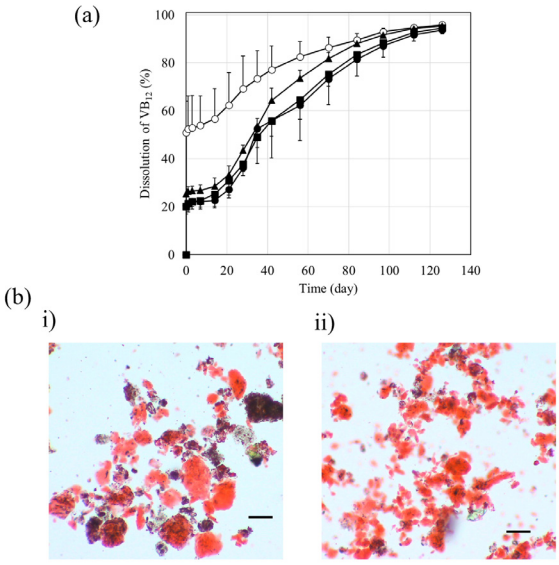

Figure 2. Effect of the addition of small balls (ϕ3 mm) on the properties of microspheres. (a) *In vitro* VB_{12} release; ●: 4 large and 4 small balls (sample No.: #5); ■: 4 large and 10 small balls (sample No.: #6); ▲: 4 large and 15 small balls (sample No.: #7); ○: 4 large balls alone (sample No.: #8). The data represents mean ± S.E. (n = 3 batches). **(b)** Optical micrograph of microspheres fabricated using *i*) 4 large balls (ϕ10 mm) alone and *ii*) 4 large (ϕ10 mm) and 10 small (ϕ3 mm) balls. The sample were dispersed in soybean oil. The bar indicates 50 µm. Resomer®RG502 was used as PLGA and 10% VB_{12} was the loaded drug. The processing time was 12 h.

20% drug loading on drug release profiles. The results are shown in Figure 1. The fabrication was performed using 20% VB_{12} as a loaded drug and 4 large balls (ϕ = 10 mm) as media. The result showed that processing time reduced the initial burst (release rate at 1 h). For the microspheres processed for 12 h, the initial burst was 34.2 ± 9.5%. Ball milling of PLGA without VB_{12} did not provide microparticles after over 30 min of processing time, resulting in a large clump.

The effect of small (ϕ = 3 mm) and large (ϕ = 10 mm) balls on the amount of VB_{12} released from microspheres were evaluated. Figure 2a shows VB_{12} release from microspheres loaded with 10% VB_{12} prepared by the dry fabrication method. Although large balls alone provided large initial burst, the combination of large and small balls reduced the initial burst. Microspheres prepared using large balls alone showed poor content uniformity, according to microscopic observation. On the contrary, microspheres fabricated using large and small balls showed good uniformity (Figure 2b). The effect of drug loading on drug release profiles is shown in Figure 3. Drug loading affected the initial burst, thus contributing to the drug release profile. Drug loading at 10%, 20%, and 30% resulted in initial bursts of 25.2 ± 1.9%, 15.6 ± 4.2%, and 58.2 ± 3.0%, respectively. FT-IR analysis shows that the any peaks of VB_{12} and PLGA did not shift in the microspheres (Figure 4). The peaks of the VB_{12}-related substances did not increase, and the unknown peaks were not generated during the dry fabrication procedure (Table 2).

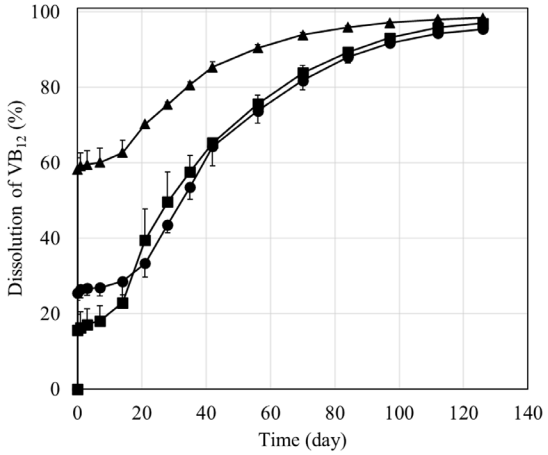

Figure 3. Effect of VB_{12} loading on *in vitro* VB_{12} release from the microspheres fabricated by the dry fabrication method. Drug loading was ●: 10% (sample No.: #7); ■: 20% (sample No.: #9); ▲: 30% (sample No.: #10). The microspheres were fabricated under the following conditions: media, 4 large (ϕ10 mm) and 15 small balls (ϕ3 mm); PLGA, Resomer®RG502. The processing time was 12 h. The data represent mean ± S.E. (n = 3 batches).

3.2. Comparison of properties of microspheres fabricated by the dry fabrication and those prepared by the conventional method

We investigated the comparison of morphological feature, particle size, encapsulation efficiency and drug release profiles of microspheres fabricated by the dry fabrication method and the conventional o/w emulsion solvent evaporation. For the morphological feature, the

conventional method provided the spherical feature. The dry fabrication method provided the aggregates gathered by several particles (Figure 5a). For the particle size distribution, the microspheres fabricated by both methods had the injectable particle size, not larger than 150 μm that is specified for suspensions for injection by the Japanese Pharmacopoeia (Figure 5b, Table 3). For encapsulation efficiency, the dry fabrication method did not loss the drug during the fabrication process, however, encapsulation efficiency by the conventional method was 36.4 ± 1.8% (Table 3). The drug release was equivalent between the microspheres fabricated by both methods (Figure 5c).

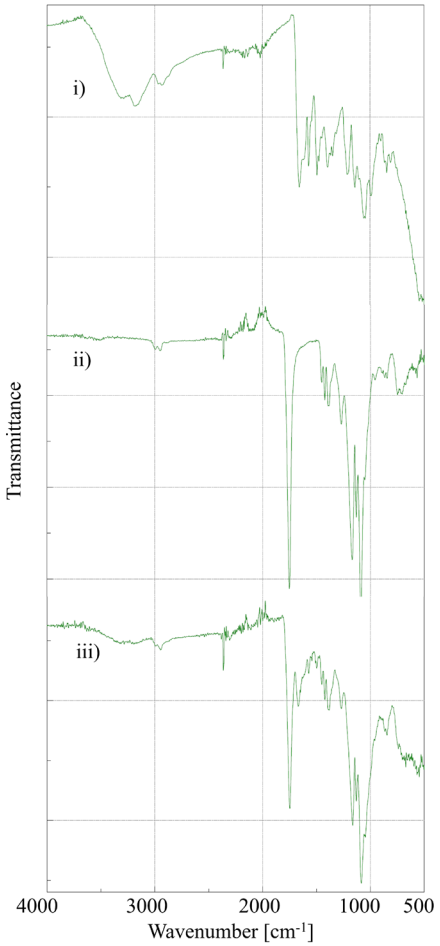

Figure 4. FT-IR analysis. *i*) VB_{12} powder, *ii*) PLGA powder, *iii*) microspheres fabricated by the dry fabrication method (sample No.: #4). The microspheres were fabricated under the following conditions: media, 4 large (ϕ10 mm) and 15 small (ϕ3 mm) balls; PLGA, Resomer®RG502; loaded drug, 20% VB_{12}; processing time 12 h.

3.3. Compatibility of the dry fabrication method with PLGA from various manufacturers

We evaluated the compatibility of the dry fabrication method with PLGA from various manufacturers under the following conditions: lactic acid: glycolic acid ratio, 50:50; inherent viscosity midpoint, 0.2 dl/g. The drug release profiles are shown in Figure 6. The order of the initial burst rate was as follows: PLGA5020 (Wako) > Resomer®RG502H (Sigma-Aldrich) > Resomer®RG502 (Sigma-Aldrich) > PURASORB®PDLG5002 (Corbion). The duration of drug release was in the order of the microspheres prepared by using Resomer®RG502 (Sigma-Aldrich) > Resomer®RG502H (Sigma-Aldrich) > PLGA5020 (Wako) > PURASORB®PDLG5002 (Corbion). With Resomer®RG502 (Sigma-Aldrich), the drug release lasted for 126 days.

4. Discussion

In general, in the process of ball milling, exothermic heat is generated by the collision between balls and samples, thus inducing the local melting of samples. The occurrence of melting in the process of amorphization by ball milling has been reported (*11*). Local melting at points of impact can induce binding between particles. Although the PLGA used in this study did not have a melting point owing to its amorphous state, binding between particles was considered to occur approximately at a softening point of 50-60°C. In contrast, VB_{12} is known to have a high melting point (> 300°C). VB_{12} particles with higher melting points are considered to be attached and bound by softened PLGA particles. PLGA particles with VB_{12} attached were pulverized and aggregated during repeated milling. Therefore, VB_{12} particles at a smaller proportion should be encapsulated

Table 2. Evaluation of VB_{12} related substances increase by the dry microsphere fabrication process

	Cyanocobalamin (%)	Related Substances (%)													
Retention time (min)	6.78	(Total)	3.14	3.49	3.70	4.03	4.89	5.33	5.49	5.79	6.22	8.07	9.42	10.07	11.19
VB_{12} PBS solution	97.37	2.63	0.06	0.04	0.12	0.01	0.05	0.17	0.26	0.22	0.09	0.06	0.98	0.26	0.30
Extracted solution from VB_{12} PBS solution	97.71	2.29	0.01	0.03	0.07	0.03	0.01	0.10	0.40	0.20	0.09	0.08	0.79	0.24	0.25
Extracted solution from VB_{12} loaded microspheres*)	97.36	2.64	0.04	0.03	0.11	0.01	0.01	0.05	0.47	0.22	0.10	0.06	0.96	0.28	0.30

*)The microspheres were fabricated under the following conditions: media, 4 large (ϕ10 mm) and 15 small balls (ϕ3 mm); process time 12h ; PLGA, Resomer®RG502; drug loading 10%.

by PLGA at a larger proportion in the microspheres. With one cycle consisting of milling for 30 min and resting for 5 min, the total milling time required to encapsulate ca.85% VB_{12} particles may be 12 h for Resomer® RG502, judging from the initial burst of 15% in the 20% VB_{12}-loaded Resomer® RG502 microspheres. From our preliminary study, approximately 15% initial burst were considered to be the lower limit, which indicated that there was no difference in the initial burst between VB_{12}-loaded microspheres fabricated through 12 and 18 h of total milling (data not shown). This initial burst rate was also equivalent to that obtained with the conventional fabrication method, indicating that the initial burst of VB_{12} from Resomer® RG502 microspheres obtained through 12 h of milling was due to the properties of the polymer and the size of the microspheres, not due to the fabrication method. The subsequent release after the initial burst was also equivalent to that obtained with the conventional method.

In general, the risk for drug degradation by exothermic heat due to collision is known as a precaution point in a milling process. This may become a major disadvantage of the dry fabrication method using a ball mill. In this study, however, VB_{12} degradation and interaction between VB_{12} and PLGA were not detected by HPLC and FT-IR analyses, respectively.

The dry fabrication method showed considerable differences from the conventional method in terms of encapsulation efficiency and microsphere morphology.

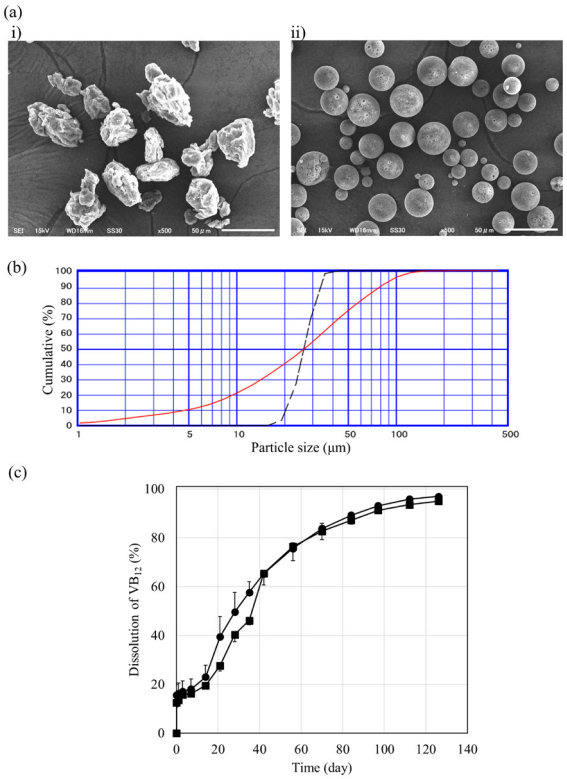

Figure 5. Comparison of microsphere properties between the dry fabrication method and the conventional o/w emulsion solvent evaporation method. (a) Scanning electron micrograph of microspheres fabricated by the *i*) dry fabrication method (sample No.: #9) and *ii*) conventional method (sample No.: #11). **(b)** Size distribution of microspheres fabricated by the dry fabrication method (sample No.: #9); solid line and conventional method (sample No.: #11); dashed line. **(c)** *In vitro* VB_{12} release from the microspheres fabricated by the dry fabrication method (sample No.: #9) (●) and conventional method (sample No.: #11) (■). The data represent mean ± S.E. (n = 3 batches). The microspheres were fabricated under the following conditions: media, 4 large (ϕ10 mm) and 15 small (ϕ3 mm) balls; PLGA, Resomer®RG502; loaded drug, 20%VB_{12}; processing time 12 h.

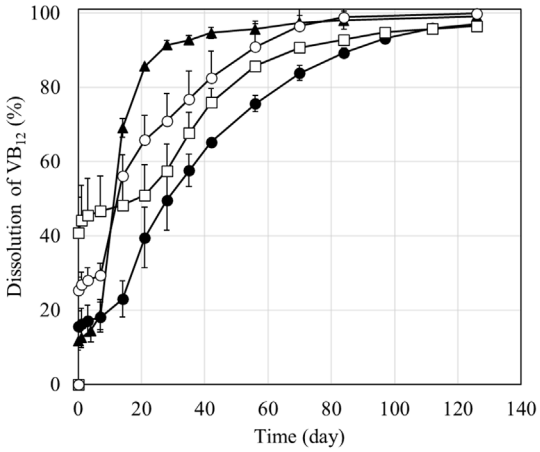

Figure 6. Compatibility of the dry fabrication method with PLGA from various manufacturers. *In vitro* VB_{12} release from the microspheres fabricated using Resomer®RG502 (sample No.: #9) (●), PURASORB®PDLG5002 (sample No.: #12) (▲), Resomer®RG502H (sample No.: #13) (○), and PLGA5020 (sample No.: #14) (□). The microspheres were fabricated under the following conditions: media, 4 large (ϕ10 mm) and 15 small (ϕ3 mm) balls; PLGA, Resomer®RG502; loaded drug, 20%VB_{12}; processing time 12 h. The data represent mean ± S.E. (n = 3 batches).

Table 3. Comparison of particle size and encapsulation efficiency of the microspheres fabricated by the dry fabrication and conventional methods

Sample No.	VB_{12} loading (%)	Fabrication Method	Particle size (μm)				Encapsulation efficiency (%)
			Mean	D_{25}	D_{50}	D_{75}	
#7	10	Dry fabrication method	40.9 ± 3.6	22.7 ± 2.9	43.5 ± 2.4	88.6 ± 5.8	95.8 ± 2.6
#9	20	Dry fabrication method	27.4 ± 6.1	14.4 ± 5.2	28.5 ± 8.0	66.4 ± 9.6	104.1 ± 5.0
#10	30	Dry fabrication method	32.0 ± 6.0	16.3 ± 5.0	41.1 ± 8.8	83.3 ± 13.5	95.4 ± 2.1
#11	20	Conventional method*)	26.2 ± 0.1	22.9 ± 0.1	26.3 ± 0.1	30.1 ± 0.1	35.3 ± 1.6

*)o/w emulsion solvent evaporation method. Data represents Mean ± S.E. (n = 3 batches).

Regarding encapsulation efficiency, the dry fabrication method showed an advantage over the conventional method. In the conventional o/w emulsion solvent evaporation method, leakage of a water-soluble drug into the external water phase during the fabrication process is known to be a major disadvantage. In contrast, there was no risk of drug leakage in the dry fabrication method owing to the solvent-free nature of the method. However, there were some precaution points on encapsulation with the dry fabrication method. One is the affinity balance between PLGA particles, drug particles, and materials used in the mill. Drug and PLGA particles can be attached on the inner surface of the mill. If there is a difference in the degree of attachment to the inner surface between drug and PLGA particles, excess amount of the component attached to the inner surface can be unused for microencapsulation, thus affecting the encapsulation efficiency of the drug into the microspheres. In this study, we used balls and a pot from agate for ball milling. In the microspheres fabricated by the dry fabrication method in this study, the observed VB_{12} content in microspheres was in agreement with the theoretical VB_{12} content; hence, the material appeared to be suitable for the dry fabrication of VB_{12}-PLGA microspheres. If there was a difference between the observed and theoretical drug concentrations, the materials should be further validated for suitability for the aimed microspheres.

Another precaution point is the content uniformity of each particle. Microsphere fabrication by using 4 large balls (ϕ10 mm) resulted in a mixture of high and low particle content. At the end of the fabrication process, most powders stuck on the top inner part of the pot, and the powder in the pot wall next to the sticking matter was mainly PLGA particles. This finding indicated that mixing did not proceed well. Uniformity is greatly related to the mixing process of the loaded particles during the ball milling. The large ball media generate a large space between balls or between balls and the pot wall. It is difficult for the powder stuck in the space to be stirred. To improve mixing, small ball media were added to fill the space. Although it is known that the close-packed structure provides minimum space, such close-packed composition of ball media, such as the five-sized particles, was reported to facilitate particle size reduction. Because the excess reduction of particle size is inconvenient for microencapsulation, we attempted to improve uniformity using volume ratios of small and large balls of up to 1/10. The addition of a small number of small balls improved content uniformity between particles both microscopically and macroscopically. However, further addition of small balls did not result in more improvement. These results indicated that the addition of a small number of small balls improved the mixing of powder during dry fabrication. As content uniformity was improved, variations in release profiles, especially in the initial burst between different manufacturing batches were also improved. Moreover, the degree of the initial burst was reduced by the addition of small balls. A possible reason is that deviation of VB_{12} encapsulation in some parts of the microspheres can occur when mixing was conducted with large balls alone, and that the deviation can be eliminated through the addition of small balls. Increasing the percentage of drug loading led to an increase in the degree of the initial burst. Hence, the overloading of VB_{12}, including unencapsulated VB_{12} particles, was considered to occur in some part of the microspheres owing to inadequate mixing, leading to increased initial burst. However, the fraction of the overloaded microspheres was decreased by increasing the degree of mixing through the addition of small balls, resulting in a decrease in the initial burst.

Another precaution in the microspheres prepared by the dry fabrication method is the needle-passing at injection. The microspheres prepared by the conventional method are complete spheres. The spherical particles are known to be the shape with the best needle-passing property. The microspheres prepared by the dry fabrication method were not spheres. Although the microspheres showed good needle-passing property in this study, they are at risk of showing a weak needle-passing property. An effort to round the particles prepared by ball milling has been reported in the field of mechanical alloying; polymer particles characterized by an irregular shape were sprayed into the thermal chamber to round the particles (*12*). Although thermal rounding is a promising technique for rounding the particles, considerable attention should be paid to the thermal stability of the encapsulated drug.

The compatibility of the dry fabrication with PLGA from various manufacturers also is an important factor in the practical aspect of manufacture. In this study, we evaluated PLGA obtained from three manufacturers and showing equivalent inherent viscosity. PLGA is classified into two types according to the terminal carboxyl group: alkyl ester capped PLGA and free carboxylic acid PLGA. Microspheres prepared using alkyl ester capped (Resomer®RG502) and free carboxylic acid (Resomer®RG502H) showed equivalent initial bursts, although they also showed some differences in drug release profiles. This indicated that the feasibility of the dry fabrication method was high and independent of the end capping of PLGA. On the contrary, microspheres prepared using PLGA from different manufacturers showed great differences in the initial burst. This indicated that the feasibility of the dry fabrication may be dependent on the brand of the PLGA used.

The present study in the 150 mg scale indicated the usefulness of the dry fabrication method for injectable PLGA microspheres. At the early stage of new drug research, only a small amount of candidate compounds is often available. In such a situation, because a new chemical entity-loaded, long-acting, injectable formulation could not be fabricated by a conventional

method in terms of applicable minimum scale and drug loss, it is difficult to evaluate its pharmacologic effectiveness. Its availability in a small scale and its low drug loss in the manufacturing process are the advantages of the dry fabrication method. However, scale-up ability is an important point to be clarified for commercial manufacturing in the future. Another related theme is the optimization of powder volume rate to the pot volume, which affects the productive efficiency. Thus, further studies are needed to evaluate these points.

In conclusion, the dry fabrication method can be an alternative method for fabricating long-acting injectable formulations, as it showed an advantage of low drug loss during the fabrication process. In this study, only one drug, VB_{12}, was used to evaluate the feasibility of the dry fabrication method. Clarifying the properties of drugs that are suitable for this method is important to advance the studies on the dry fabrication method. We are planning to investigate the drug properties suitable for the dry fabrication method.

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Original Article

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Therapeutic effects of sertraline on improvement of Ovariectomy-induced decreased spontaneous activity in mice

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SUMMARY We have already reported that ovariectomized (OVX) rats reduced the spontaneous activity during the dark period due to the decease of serotonin release in the amygdala. In this study, we examined the potential of sertraline, a selective serotonin reuptake inhibitor, on the recovery of less spontaneous activity seen in mice with OVX-induced despair-like behaviors. Female 9-week old ICR mice were underwent either OVX or sham surgery. Sertraline (10 mg/kg/day, *s.c.*) or saline were started to administer to each group for 8 weeks (6 times/week) from the 8th week after OVX. Each spontaneous activity of mouse was evaluated during the dark period (19:00-07:00) using an infrared sensor. Moreover, mRNA expression levels of tryptophan hydroxylase (TPH) and X-box binding protein 1 (XBP1) were measured in the hippocampus and prefrontal cortex using by a real-time PCR method. We found out that the OVX-induced despair-like behaviors were improved by the continuous administration of sertraline. After treatment of OVX, our real-time PCR data showed that sertraline significantly suppressed the upregulation of XBP1 expression levels in both hippocampus and prefrontal cortex, although this suppression of the downregulation of TPH expression levels was seen in only hippocampus. These results suggest that sertraline improves the decrease in spontaneous activity induced by OVX assessed by the hippocampus suppressing decreased serotonin synthesis in the serotonergic neuron.

Keywords Sertraline, ovariectomized mice, spontaneous activity, hippocampus, serotonergic neuron, tryptophan hydroxylase

1. Introduction

There are gender differences in the incidence of depressive symptoms and mood disorders, and it has been reported that the lifetime prevalence of depression in women is about twice that in men (*1*). Furthermore, women often exhibit anxiety, emotional instability, and depression during the menstrual cycle in the case of premenstrual syndrome and psychiatric symptoms such as anxiety, depression, fear, and fatigue during menopause (*2*). It is thought that estrogen, a female hormone, may be involved in the onset of these specific women's psychiatric symptoms, although the causes of these symptoms induced by changes in estrogen levels have not been elucidated, yet. There are many reports that estrogen administration inhibits anxiety and depressive symptoms in animal experiments, although there is also a conflicting report that estrogen increases symptoms (*3*); therefore, a consensus has not been reached. In clinical practice, hormone replacement therapy (HRT) is used as a treatment of menopause disorder, but the efficacy of HRT is limited for treating vasomotor symptoms such as hot flashes and sweating (*4*), and there is no effective treatment of depression. Furthermore, estradiol administration has been reported to increase the risk of breast and endometrial cancer (*5,6*), and there is a need for the development of treatments of estrogen-dependent psychiatric symptoms. In particular, psychiatric symptoms during menopause are likely to develop into major depression, which is one of the factors contributing to the high suicide rate among older women (*7*); thus, prompt elucidation of the mechanism of estrogen-dependent psychiatric symptoms is desirable.

It has been reported that monoamine neurotransmitters

in the brain are involved in the onset of symptoms such as depression and anxiety due to decreased estrogen levels, suggesting involvement of the serotonergic neuronal system (*8,9*). We also reported that the significant reduction of estradiol after ovariectomy (OVX) suppresses the spontaneous activity during the dark period and that brain serotonin is involved in such effect in rats (*10*). In addition, we previously reported that the specific serotonin uptake inhibitor (SSRI), fluvoxamine, counteracts the decrease in spontaneous activity in an OVX rat model (*11*).

At present, the efficacy of SSRIs and serotonin and norepinephrine reuptake inhibitors (SNRIs) has been clinically demonstrated for the main treatment for symptoms of depression during menopause (*12*). Furthermore, it is well known that there are gender differences in the therapeutic response to antidepressants, and women have been reported to show better response to sertraline, a typical SSRI, than the tricyclic antidepressant imipramine (*13*). In addition, the results of a non-clinical study showed that female transgenic mice deficient in aromatase are more responsive to sertraline than males (*14*). Thus, sertraline may be highly effective against the female depression-like symptoms. Sertraline is widely known increasing the serotonin concentration in synaptic cleft by selectively inhibiting serotonin reuptake, while Kim *et al.* reported that long-term administration of sertraline increases tryptophan hydroxylase (TPH) mRNA of rate-limiting enzyme for serotonin synthesis (*15*). In addition, the expression of transcription factor X-box protein 1 (XBP1) increases in stress-induced rats and is associated with the endoplasmic reticulum (ER) stress response (*16*), suggesting a correlation between depression and ER stress response.

In the present study, we evaluate whether sertraline, which is a potential clinical treatment of estrogen-dependent psychiatric symptoms, recover the behavioral changes in OVX model mice. In order to resolve this questionnaire, we investigated both gene expression levels of TPH, which is the rate-limiting enzyme for serotonin synthesis, and XBP1, which is involved in the ER stress response, in OVX mice.

2. Materials and Methods

2.1. Animals and rearing conditions

A total of thirty-two ICR female mice (8 weeks old, Japan SLC, Shizuoka, Japan), which had undergone one week of preliminary rearing, were used for the study. The breeding environment was set to room temperature (24 ± 1°C), 12-h light/dark cycles (light period: 07:00-19:00; dark period: 19:00-07:00). Mice were allowed free access to a standard rodent diet (Labo MR stock, Nosan Corporation, Kanagawa, Japan) and water during the entire experimental period. The present study was approved by the Animal Experiment Committee of the Yokohama University of Pharmacy (approval number: 2017-021), and care was taken to the welfare of laboratory animals.

2.2. OVX and administration

After 1 week of preliminary rearing, the animals were divided into 4 groups to minimize the difference in weight. Under inhalation anesthesia with isoflurane, one of the groups underwent sham surgery (sham group), and the other 3 groups underwent OVX. Drug administration was initiated 8 weeks after OVX. Eight weeks after OVX, sertraline hydrochloride (Tokyo Chemical Industry, Tokyo, Japan) was administered (10 mg/kg *s.c.*) to the OVX + sertraline administration group, and β-estradiol (Sigma Sigma-Aldrich St. Louis, MO, USA) was administered (20 μg/kg *s.c.*) to the OVX + β-estradiol administration group. The Sham and OVX groups were administered saline by *s.c.*. Administration was performed at 10 mL/kg, 5 days per week, over a period of 8 weeks.

2.3. Measurement of uterine weight

Eight weeks after administration of test drugs (16 weeks after OVX), the mice were euthanized, the uterus was removed, and the weight of the uterus was measured.

2.4. Measurement of spontaneous activity

After 8 and 16 weeks following OVX, the mice were placed in individual cages using a spontaneous activity-measuring device (NS-AS01, Neuroscience, Tokyo, Japan), and the amount of activity per hour was measured with an infrared sensor. The measurement was performed during the 12-h dark period (19:00-07:00), and the total activity was compared.

2.5. Open field test

Eight weeks after administration, the behavior of the mice was observed for 10 min using an open field test device. The device features a 50 cm^2 shape at the bottom and a wall height of 30 cm; the bottom and side walls are made of gray plastic. Each mouse was released at the center of the device, their behavior was observed for 10 min, and the track length and duration were then measured. After each test, any excrement was removed, and the device was wiped clean with 10% ethanol. The square at the bottom of the device was divided into central (25 cm × 25 cm) and outer areas, and the ratio of the track length to the central area and the duration was calculated.

2.6. TPH, serotonin transporter (5-HTT), and XBP1 mRNA expression levels

To extract total RNA, Isogen (Nippon Gene, Tokyo,

Japan) was added to the hippocampus and frontal lobe specimens extracted from each mouse and homogenized with POLYTRON PT 1300 D (Central Scientific Commerce, Tokyo, Japan). Chloroform (Nacalai Tesque Inc., Kyoto, Japan) was added to the homogenized samples, the samples were centrifuged, and the supernatant was collected in a new tube. Isopropanol (Nacalai Tesque Inc.) was added to the supernatant, and the precipitate was collected by centrifugation. The precipitate was then resuspended in sterile water, the RNA concentration was measured, and cDNA was synthesized using the SuperScript VIRO cDNA Synthesis kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) protocol, with an incubation for 10 min at 25°C, 60 min at 42°C, and 5 min at 85°C. For real-time PCR, LightCycler 480 II (F. Hoffmann-La Roche, Basel, Switzerland) was used with a primer pair for each marker (Table 1), and the TaqMan probe method was followed for 40 to 50 cycles, where each cycle consisted of 95°C for 10 min, 95°C for 10 s, and 60°C for 20-30 s.

2.7. Statistics

Data are shown as the mean ± standard error. Comparison of the data between groups was performed using Fisher's protected least significant difference (PLSD) method. Stat View (SAS institute Inc., version 5.0) was used as a statistical software. The significance level was set as $p < 0.05$ or $p < 0.01$.

3. Results

3.1. Effects on the uterus

The weights and entities of representative uteri, 16 weeks after OVX, are shown in Figure 1. The uterine weight significantly decreased in the OVX group in comparison with the sham group. The decrease in uterine weight induced by OVX was significantly mitigated by β-estradiol administration, but no effect on the uterus was observed in the sertraline administration group.

3.2. Spontaneous activity

The results of the measurement of spontaneous activity during the dark period, 8 weeks after OVX, revealed that there was no difference in circadian rhythm. However, there was a significant decrease in total activity during the dark period in the OVX group compared with the sham group (Figure 2a).

Eight weeks after administration (16 weeks after OVX), there was no difference in spontaneous activity circadian rhythm, consistent with the observations 8 weeks after OVX. In contrast, the OVX group showed a significant decrease in total activity during the dark period in comparison with the sham group. The decrease in total activity in the OVX group was significantly improved in the sertraline and β-estradiol administration groups (Figure 2b).

3.3. Open field test

The results of the open field test revealed that there was no significant difference in the track length across all areas in the device between the different administration groups; however, the OVX group showed a decreasing trend compared with the sham group, and an improvement was observed in the sertraline and β-estradiol administration groups (Figure 3b). The ratio of the track length from the outer area to the center increased significantly in the OVX group compared with the sham group, and a tendency for the increase to be reduced was observed in the sertraline and β-estradiol administration groups (Figure 3c). The ratio of duration in the outer area to the central part was significantly increased in the OVX group in comparison with the sham group (Figure 3d). Moreover, the increase led to a significant recovery in the sertraline and β-estradiol administration groups (Figure 3d).

3.4. mRNA expression levels of TPH, 5-HTT, and XBP1

Changes in mRNA expression levels in the mouse

Table 1. List of all primers used for real-time PCR

Gene	Universal Probe Livrary Probe No.	Sequence (5'→3')
GAPDH	#9	Forward:agcttgtcatcaacgggaag Reverse: tttgatgttagtggggtctcg
TPH	#21	Forward: cacagttcagatcccctctaca Reverse: gaacgtggcctaggagttca
5-HTT	#62	Forward: acctggacactccattccac Reverse: cctggagtccctttgactga
XBP1	#39	Forward: gctggatcctgacgaggtt Reverse: gccaccagccttactccac

GAPDH: *Mus musculus* glyceraldehyde 3-phosphate dehydrogenase, TPH: *Mus musculus* tryptophan hydroxylase, 5-HTT: *Mus musculus* serotonin transporter, XBP1: *Mus musculus* X box-binding protein 1.

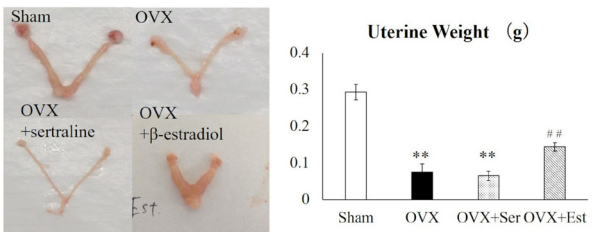

Figure 1. Effects of β-estradiol and sertraline on the uterine weight at 16 weeks. The images are of representative uteri from the different treatment groups. The data are shown as the mean ± standard error. $^{**}p < 0.01$ compared with the sham group. $^{\#\#}p < 0.01$ compared with the OVX group. Sham: sham surgery group; OVX: ovariectomy surgery group; Est: β-estradiol administration group; Ser: sertraline administration group.

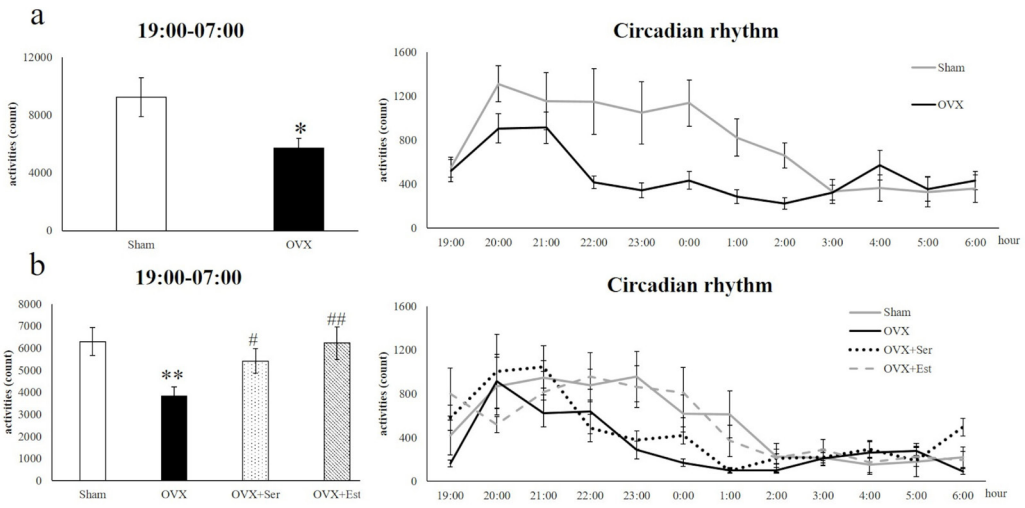

Figure 2. Spontaneous locomotor activities of mice in the sham, OVX, OVX with β-estradiol, and OVX with sertraline groups. (a) Eight weeks after ovariectomy and **(b)** 8 weeks after administration. The amount of spontaneous locomotor activities was measured from 15:00 to 10:00, and the amount of activity for 12 hours from 19:00 to 7:00 was calculated. The data are shown as the mean ± standard error. $^{**}p < 0.01$ or $^{*}p < 0.05$ compared with the sham group; $^{\#\#}p < 0.01$ or $^{\#}p < 0.05$ compared with the OVX group. Sham: sham surgery group; OVX: ovariectomy surgery group; Est: β-estradiol administration group; Ser: sertraline administration group.

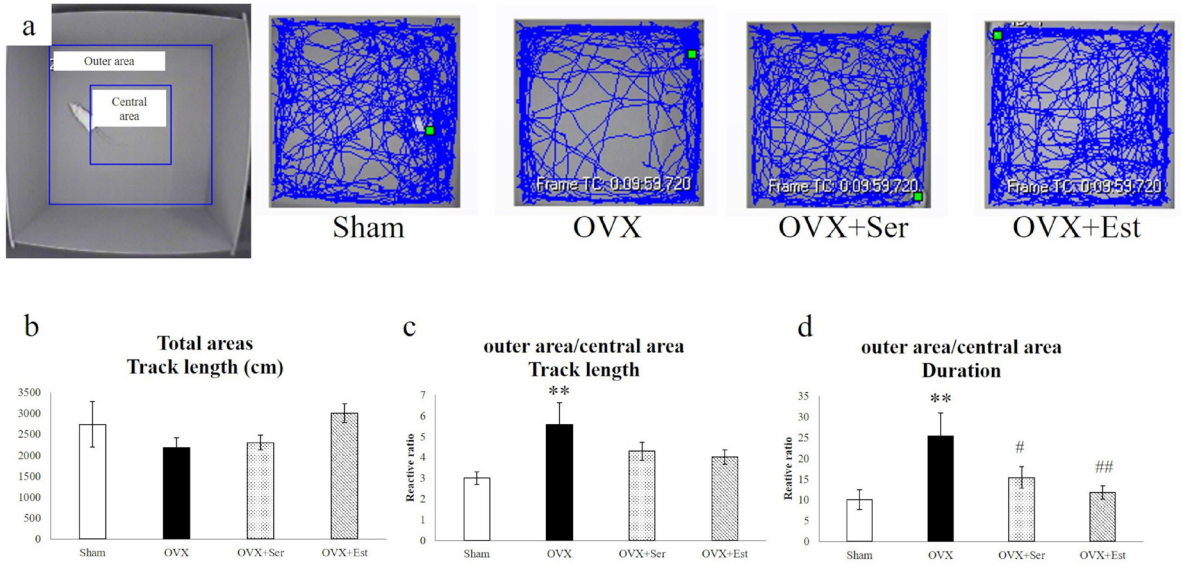

Figure 3. Open field test of mice in the sham, OVX, OVX with β-estradiol, and OVX with sertraline groups. (a) The images of representative from the different treatment, **(b)** Track length in total areas, **(c)** the ratio of the track length (outer area / central area) and **(d)** the ratio of the duration (outer area/central area). The data are shown as the mean ± standard error. $^{**}p < 0.01$ compared with the sham group; $^{\#\#}p < 0.01$ or $^{\#}p < 0.05$ compared with the OVX group. Sham: sham surgery group; OVX: ovariectomy surgery group; Est: β-estradiol administration group; Ser: sertraline administration group.

hippocampus and frontal lobe were examined by real-time PCR. In the hippocampus, the *TPH* gene expression level was significantly decreased in the OVX group compared with the sham group; this decrease was significantly counteracted in the sertraline administration group but no effect was noted in the β-estradiol administration group (Figure 4a). No significant difference in the *TPH* gene expression level in the frontal lobe was noted (Figure 4b). While the *5-HTT* gene expression levels showed a decreasing trend in both the hippocampus and frontal lobe in the OVX group in comparison with the sham group, no significant differences were noted, and the β-estradiol administration group showed a significant increase compared with the OVX group. The *XBP1* gene expression levels were significantly higher in the hippocampus and frontal lobe in the OVX group compared with the sham group; this increase was significantly diminished in the sertraline

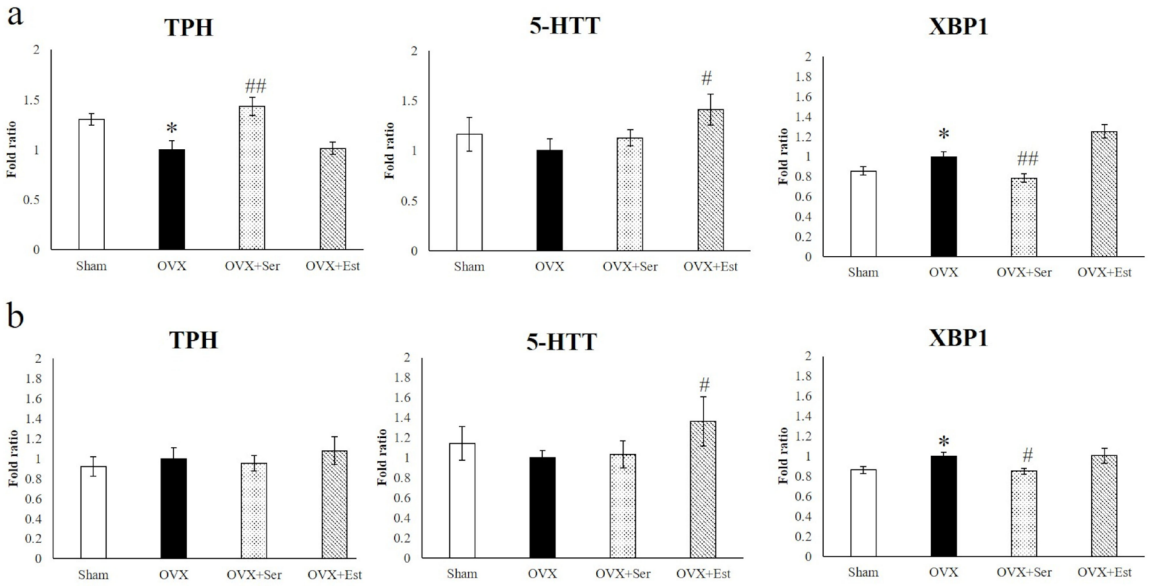

Figure 4. Effects of sertraline on the mRNA expression levels of TPH, 5-HTT, and XBP1. Expression levels in the **(a)** hippocampus and **(b)** prefrontal cortex. The data are shown as the mean ± standard error. $^{*}p < 0.05$ compared with the sham group; $^{\#\#}p < 0.01$ or $^{\#}p < 0.05$ compared with the OVX group. Sham: sham surgery group; OVX: ovariectomy surgery group; Est: β-estradiol administration group; Ser: sertraline administration group.

administration group, but no effect was observed in the β-estradiol administration group (Figure 4).

4. Discussion

4.1. Influence on the uterine

The present study focused on the effects of sertraline, a SSRI, on psychiatric symptoms specific to women, using OVX model mice. In current clinical practice, HRT is mainly performed for the menopausal disorders in women. However, it has been reported that estrogen administration induced uterine cancer and breast cancer (*5,6*). In the present study, the uterine of OVX model group showed a significant atrophy compared to that of the sham group. Moreover the atrophy was significantly enlarged after administration of β-estradiol (Figure 1). And also our study found out the hyperplasia of uterine in the β-estradiol treatment group. The previously reported paper supported that the continuous administration of β-estradiol cause the hyperplasia in rats (*17*). However, administration of sertraline showed no effect on the uterus, pathologically. These results suggest that administration of sertraline does not cause any risks to the uterus but not β-estradiol administration.

4.2. Behavioral experiments

We previously reported that the spontaneous activity in OVX model rats decreased during the dark period as measured for 24 hours (*10*). Similarly, the present study using mice showed a significant decrease in spontaneous activity during the dark period was observed for all OVX mice (n = 24) 8 weeks after OVX (Figure 2a). This data also showed that the decreased estrogen due to OVX reduced the amount of activity during the active period (dark period) in mice. We have reported that the treatment of fluvoxamine, another SSRI, just after OVX significantly inhibited the reduction of spontaneous behavior in rats induced by OVX (*11*). Saloua *et al.* reported that administration of sertraline just after OVX induced antidepression-like behavior (*18*). These reports have shown that SSRIs have been administered at the same time as OVX surgery and have a preventive effect. However, in clinically, SSRIs are clinically used after symptoms of depression during menopause was occurred. In this study, β-estradiol and sertraline administrated 8 weeks after OVX and its therapeutic effect was examined. The result showed that the decrease in spontaneous activity due to OVX was significantly recovered by the administration of either β-estradiol or sertraline for 8 weeks (Figure 2b). Few reports are available on administration from a state in which the amount of the spontaneous activity was reduced by OVX, and the results of this study are new findings. These results suggest that the recovery of decreased spontaneous behavior induced by OVX relates to serotonergic neuron.

In the open field test, OVX showed a significant increase in the amount of outer area activity (track length and duration) (Figure 3c). Administration of either β-estradiol or sertraline significantly suppressed decrease of duration in outer area by OVX (Figure 3d). The open field test is one of the widely used methods for measuring anxiety-related emotional behavior (*19*).

The mouse prefers to stay adjacent to the wall and move around the outer area, which is described "thigmotaxis". It is reported thigmotaxis significantly increases as anxiety levels rise in mice (*19,20*). This study showed that sertraline suppress increase of thigmotaxis due to OVX. These results suggest that sertraline improved anxiety-like behavior in OVX model mice.

In the present study, sertraline improved the behavioral changes caused by OVX in both the spontaneous activity and open field tests. As a result, sertraline appeared to ameliorate psychiatric symptoms such as anxiety, depression, and fatigue induced by decreased estrogen levels. Benmansour *et al.* also reported that sertraline administration, initiated at the same time as OVX surgery in OVX model rats, resulted in improved behavioral changes induced by OVX after 2 weeks of administration (*21*). In the present study, we investigated sertraline administration after decreased spontaneous activity due to OVX, and the results revealed that sertraline has a therapeutic effect on estrogen-dependent behavioral changes in OVX model mice.

4.3. mRNA levels for related the serotonergic cell functions in the brain

The involvement of brain monoamine neurotransmitters such as noradrenaline and serotonin has been reported in the case of psychiatric symptoms such as depression and anxiety induced by decreased estrogen levels (*22,23*). We previously showed that serotonin release in the amygdala is decreased in OVX model rats and that the serotonin nervous system is involved in the decrease in spontaneous activity induced by OVX (*10,11*). In the present study, the *TPH* gene expression level in the hippocampus was decreased by OVX, and sertraline significantly inhibited this decrease (Figure 4a). However, the *5-HTT* gene expression levels did not differ between the sham and OVX groups. TPH is a rate-limiting enzyme for serotonin synthesis, catalyzing the conversion of l-tryptophan to 5-hydroxytryptophan. It is well known that sertraline increases the serotonin concentration in the synaptic cleft by selectively inhibiting serotonin reuptake, and Kim *et al.* report that long-term administration of sertraline increases TPH mRNA and protein levels (*15*). The results of the present study are consistent with this report, suggesting that long-term administration of sertraline may increase serotonin release by increasing serotonin synthesis.

Sertraline significantly inhibited the OVX-induced increase in the expression of the *XBP1* gene, which is involved in the ER stress response and unfolded protein response (UPR), in both the hippocampus and frontal lobe (Figure 4). ER stress has been implicated in depression-like symptoms, and it has been reported that ER stress-related gene expression levels increase in the hippocampus of rats subjected to restraint stress or electric shock (*16,24*). Moreover, the relationship between ER stress and estrogen has been reported (*25,26*), but there have been no reports on UPR in OVX model mice. In the present study, OVX significantly increased the gene expression level of *XBP1*, suggesting that a decrease in estrogen may induce an excessive ER stress response. Sertraline administration significantly inhibited the OVX-induced increase in *XBP1* gene expression. These results suggest that UPR may well be involved in improvement the OVX-induced decrease spontaneous activity by sertraline, but there are some limitations in this study. UPR pathway is under the control of the PKR-like endoplasmic reticulum kinase (PERK) pathway, inositol requiring enzyme-1-XBP1 pathway, and activating transcription factor (ATF) 6 pathway. Further studies also need to analysis of other ER stress and UPR markers such as C/EBP homologous protein, ATF6, PERK, glucose-regulated protein 78.

In conclusion, the present study demonstrated that sertraline ameliorates the behavioral changes induced by the decrease of estrogen levels and that sertraline can expect as therapeutic effects on OVX model mice. In addition, these results suggest that sertraline improves suppressing serotonin synthesis in the serotonergic neuron in the hippocampus.

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Brief Report

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Positron-emission-tomography in tubercular lymphadenopathy: A study on its role in evaluating post-treatment response

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SUMMARY Lymph node tuberculosis is one of the most common forms of extrapulmonary tuberculosis worldwide. The study aimed to evaluate the role of positron emission tomography-computed tomography (PET-CT) in determining post-treatment response in lymph node tuberculosis. A PET-CT was done in all treatment naïve tubercular lymphadenitis adults at baseline and after six months of therapy. The post-treatment clinical response was compared with the metabolic response on PET-CT. Of the 25 patients with tubercular lymphadenitis, 9/25 patients showed a complete metabolic response (CMR) at six months, while 16 patients had a partial metabolic response (PMR). All patients with CMR had a good clinical response. However, discordance between clinical and PET findings was noticed in those with PMR. The role of PET-CT in evaluating post-treatment response in patients with tubercular lymphadenitis needs further evaluation with a larger sample size.

Keywords FDG PET-CT, lymph-node, tuberculosis

1. Introduction

Tuberculosis has affected humanity since ages and continues to be a significant global public health epidemic even today (*1*). Extrapulmonary tuberculosis (EPTB) forms a substantial proportion of the total number of cases (*2-4*). Lymph node tuberculosis (LNTB) has been reported as the most frequent form of EPTB, with an incidence of 30.8 per 100,000 population (*3*). The management of lymph node tuberculosis is a challenging ordeal as many patients continue to have persistent swelling despite the prescribed duration of treatment. Often, the endpoint of therapy is difficult to determine objectively (*4*). The present guidelines of LNTB have been mainly extrapolated from experience with pulmonary tuberculosis. The duration of treatment in tubercular lymphadenitis is usually guided by a clinical response alone. There is a need for a more accurate objective tool for evaluating response to therapy in patients with LNTB. This study aimed to determine the role of positron emission tomography-computed tomography (PET-CT) in assessing the treatment responses in patients with LNTB.

2. Materials and Methods

This is a prospective study in which treatment naïve adults (> 14 years of age) diagnosed as LNTB were enrolled in the study after taking written informed consent. Patients with significant comorbidities (malignancy, immunosuppression) were excluded from the study. Pregnant and lactating females were also excluded. An approval from the Institute Ethics Committee was obtained before the study was initiated (IECPG/384/29.06.2016). Clinical findings and baseline laboratory investigations were noted for all patients. A PET-CT scan (pre-therapy) was done at the baseline before the initiation of treatment. The treatment for lymph node tuberculosis was guided by the national guidelines for lymph node tuberculosis (*2*). A repeat PET-CT (post-therapy) was done after six months for all the recruited patients. Before the post-therapy scan was done, they were classified to have "complete clinical response (CCR)" or "partial clinical response (PCR)" based on the resolution of clinical symptomatology by a team of expert clinicians not involved in the treatment process. After the post-therapy scan was done, both the images were analyzed and reported by two nuclear medicine physicians blinded to the clinical response. Anatomical regions and the total number of sites of abnormal tracer accumulation were noted. Based on the pre-therapy scan and post-therapy scan parameters, the patients were classified to have "complete metabolic response (CMR)" and "partial metabolic response

(PMR)". The treatment was stopped in those patients with CCR. The decision to continue or stop treatment in patients with PCR was taken by the treating clinician not involved in the study.

Lymph nodes with the most intense FDG-uptake were carefully identified on both the scans. The maximum standardized uptake value (SUV-max) of tracer in the nodes was assessed using a circular region of interest. A receiver operating characteristic (ROC) curve analysis was performed to find the sensitivity and specificity of delta SUV-max (% change between the SUV-max pre-scan and post-scan) in predicting clinical response. The differences in the percentage change in SUV-max were assessed using an unpaired non-parametric Wilcoxon test. A p-value of less than 0.05 was considered significant.

Table 1. Clinical, laboratory and radiological features of the study population

	Variable	N (%)
A	Clinical Features	
1	Fever	20 (80)
2	Cough	19 (76)
3	Weight loss	15 (60)
4	Neck swelling	12 (48)
5	Abdominal pain	9 (36)
6	Chest pain	6 (24)
7	Back pain	5 (20)
B	Radiological manifestations (PET/CT)	
1	Cervical	19 (76)
2	Mediastinal	20 (80)
3	Abdominal	10 (40)
4	Pelvic	5 (20)
5	Extra-nodal	8 (40)
6	Necrotic lymphnodes	22 (88)
C	Cytopathological Diagnosis of TB	
1	Necrotizing granuloma	20 (80)
D	Microbiological Diagnosis	
1	AFB positive	3 (12)
2	CB-NAAT (Gene-Xpert) positive	9 (36)
3	MGIT Culture positive	3 (12)

3. Results and Discussion

Of the 62 patients who were screened, 37 patients were recruited for the study. After a baseline scan, 12 patients did not return for the repeat scan. A total of 25 patients were included in the final analysis. All patients were diagnosed based on the clinical and radiological features. Concurrent histopathological and microbiological evidence was present in 80% (n = 20) and 40% (n = 10) of the patients respectively. The study group consisted of 13 males and 12 females with a median age of 29.7 years (range 14-75 years). A total of 80% (n = 20) of the patients had mediastinal lymphadenopathy, while cervical and abdominal lymphadenopathy was seen in 76% (n = 19) and 40% (n = 10), respectively. Multiple lymph node sites were involved in 76% (n = 19) of the patients. In nine patients, PET demonstrated additional involvement sites, including liver, spleen, ileocecal region, pleura, pericardium, and dorsal spine. The clinical, radiological, pathological and microbiological features are summarized in Table 1.

The visual analysis of FDG/PET-CT showed a CMR in 9/25, all of whom showed a CCR (Table 2). Figure 1 shows CMR in one of the recruited patients. A PMR

Table 2. Patient characteristics, PET/CT findings and imaging and clinical outcome in 25 patients

Study No	Sex	Age	Station	Mode of diagnosis	Metabolic response	Clinical Response
1	F	74	Cervical, Abdominal	Microbiological	Partial	Complete
2	F	24	Cervical, Mediastinal, Extra-nodal	Histology	Partial	Complete
3	F	20	Cervical, Mediastinal, Extranodal	Microbiological	Complete	Complete
4	F	36	Mediastinal	Microbiological	Partial	Complete
5	M	23	Mediastinal	Microbiological	Complete	Complete
6	M	37	Mediastinal, Pelvic	Histology	Complete	Complete
7	F	32	Cervical	Microbiological	Partial	Partial
8	F	18	Pelvic, Extranodal	Radiological	Partial	Complete
9	F	23	Cervical, Mediastinal	Histology	Partial	Complete
10	M	70	Cervical, Mediastinal	Radiological	Partial	Partial
11	M	28	Mediastinal	Histology	Complete	Complete
12	M	19	Cervical, Mediastinal	Microbiological	Complete	Complete
13	F	30	Cervical, Abdominal	Histology	Complete	Complete
14	F	20	Mediastinal	Radiology	Partial	Partial
15	M	19	Cervical, Mediastinal, Abdominal, Pelvic, extra-nodal	Microbiological	Complete	Complete
16	M	30	Cervical	Histology	Complete	Complete
17	F	15	Cervical, Mediastinal, Abdominal, Pelvic	Histology	Partial	Partial
18	F	14	Cervical, Mediastinal, Abdominal, Extra-nodal	Histology	Partial	Complete
19	M	28	Cervical, Mediastinal, Abdominal, Pelvic	Histology	Partial	Partial
20	M	47	Cervical, Mediastinal, Extra-nodal	Histology	Complete	Complete
21	M	22	Cervical, Mediastinal, Abdominal, Extra-nodal	Microbiological	Partial	Partial
22	F	28	Cervical, Mediastinal, Abdominal, Extra-nodal	Histology	Partial	Partial
23	M	35	Cervical, Mediastinal, Abdominal, Pelvic, Extranodal	Microbiological	Partial	Complete
24	M	18	Cervical, Mediastinal	Histology	Partial	Complete
25	M	32	Cervical, Mediastinal, Abdominal	Microbiological	Partial	Complete

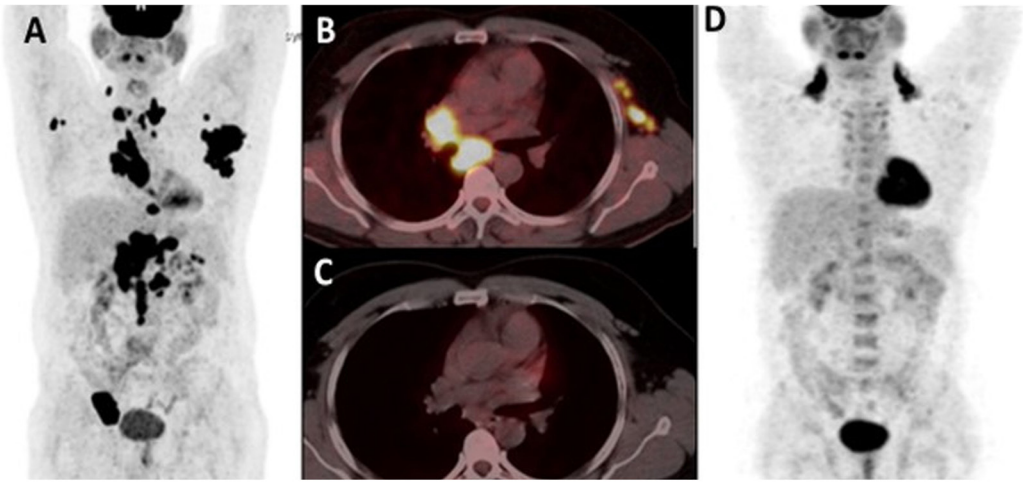

Figure 1. Before ATT showing multiple lesions of intense FDG uptake in cervical, axillary, mediastinal, abdominal and inguinal lymph nodes (A and B). After 6 month of ATT all pathological foci showed complete metabolic response (C and D).

was seen in 16/25 patients, nine of these patients showed a CCR and their treatment was stopped. The remaining seven patients with PMR and PCR were continued on therapy for a variable range of duration. All patients were followed up until the end of the study period. None of the patients with CCR (including 9 with CMR and 9 with PMR) relapsed until the end of the follow-up period. The mean duration of follow-up after completion of treatment was 127 +/- 57 days.

SUV max of lymph nodes for both the scans was computed. The percentage change in standardized uptake value (ΔSUV-max) was analyzed between baseline PET and the scan after six months of treatment. The changes in the SUV-max values were statistically significant in cervical and mediastinal lymph node stations with a p-value of 0.043 and 0.006, respectively. However, abdominal lymph nodes did not show a statistically significant change. A receiver operating characteristic (ROC) curve analysis was done to analyze the diagnostic utility of the per cent change in SUV-max as a marker of clinical response to treatment in patients with cervical/ mediastinal lymph nodes. A change in 77% or more in the SUV-max of cervical lymph nodes had a sensitivity and specificity of 75% (95%CI: 42.8-94.5%) and 100% (95%CI: 47.8-100%) respectively in predicting clinical response. A change in 52% or more in the SUV-max of mediastinal lymph nodes had a sensitivity and specificity of 75% (95%CI: 47.6-92.7%) and 100% (95%CI: 54.1-100%) respectively in predicting clinical response.

FDG PET-CT is increasingly being used for diagnosis and guiding therapeutic decisions in patients with infectious diseases. The available literature on FDG/PET-CT for assessing results and treatment response in patients with pulmonary TB is limited and suggests a good correlation between the two of them (*5*). However, there is a paucity of literature evaluating FDG/PET-CT's role in EPTB in particular. PET-CT has been hypothesized to predict the need for treatment intensification or prolonged therapeutic strategies or change in the treatment regimen (*6*). Experimental animal models have shown that FDG PET-CT activity seems to be in direct correlation to the bactericidal activity of anti-tuberculosis treatment (*7,8*).

It is pertinent to note that FDG uptake is based on the glycolytic activity in the neutrophils, lymphocytes and macrophages and represents inflammation (*9,10*). Therefore, the absence of uptake suggests a decrease in inflammation, suggesting that the patient has responded to treatment. This is the reason why in patients with no metabolic uptake in the post-therapy scans had all responded clinically. It is evident from our results that the persisting activity may not suggest active disease. In such cases, clinical assessment should be given more importance in deciding the course of action (*11-18*). Similar to a previously published study, our study showed that change in SUV-max could be used as a surrogate for predicting clinical response as well (*15*). We found that percentage decrease of 77% for cervical lymph nodes and 52% decrease for mediastinal lymph nodes had excellent specificity and considerable sensitivity. However, there is a need for a larger sample size to validate the cut-offs that we derived.

In conclusion, although PET may be useful as an adjunct to clinical response in guiding duration of therapy in some cases of tubercular lymphadenitis, its routine use as a stand-alone guide for treatment response needs to be ascertained with further studies with larger sample size.

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Multidrug treatment for COVID-19

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SUMMARY An outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, which began in Wuhan, China in December 2019, has rapidly spread all over the world. The World Health Organization characterized the disease caused by SARS-CoV-2 (COVID-19) as a pandemic in March 2020. In the absence of specific treatments for the virus, treatment options are being examined. Drug repurposing is a process of identifying new therapeutic uses for approved drugs. It is an effective strategy to discover drug molecules with new therapeutic indications. This strategy is time-saving, low-cost, and has a minimal risk of failure. Several existing approved drugs such as chloroquine, hydroxychloroquine, doxycycline, azithromycin, and ivermectin are currently in use because of their efficacy in inhibiting COVID-19. Multidrug therapy, such as a combination of hydroxychloroquine and azithromycin, a combination of doxycycline and ivermectin, or a combination of ivermectin, doxycycline, and azithromycin, has been successfully administered. Multidrug therapy is efficacious because the mechanisms of action of these drugs differ. Moreover, multidrug therapy may prevent the emergence of drug-resistant SARS-CoV-2.

Keywords hydroxychloroquine, doxycycline, azithromycin, ivermectin, COVID-19

An outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, which began in Wuhan, China in December 2019, has rapidly spread all over the world. The World Health Organization characterized the disease caused by SARS-CoV-2 (COVID-19) as a pandemic in March 2020. In the absence of specific treatments for the virus, treatment options are being examined. Drug repurposing is a process of identifying new therapeutic uses for approved drugs. It is an effective strategy to discover drug molecules with new therapeutic indications. This strategy is time-saving, low-cost, and has a minimal risk of failure. Several existing approved drugs such as chloroquine, hydroxychloroquine, doxycycline, azithromycin, and ivermectin, are currently in use because of their efficacy in inhibiting COVID-19.

Chloroquine, a drug known for its efficacy in treating malarial and autoimmune diseases such as rheumatoid arthritis and lupus erythematosus, offers promise in inhibiting SARS-CoV-2. Previous studies have revealed that it potentially has broad-spectrum anti-viral activity by increasing the pH of endosomes and lysosomes, thus preventing the process by which the virus fuses with host cells and subsequently replicates (*1*). Chloroquine is the first drug reported to have efficacy against COVID-19 in clinical studies in China (*2*). Feedback from an international meeting that took place to share experiences related to the prevention and control of COVID-19 highlighted the fact that chloroquine demonstrated significant efficacy in reducing the time to virus-negative conversion and in restabilizing body temperature (*3,4*). Hydroxychloroquine, a more tolerable derivative of chloroquine, has also been found to display potent activity against SARS-CoV-2 *in vitro* (*5*). Clinical studies in China have indicated that hydroxychloroquine can help to reduce the time until body temperature returns to normal, decrease the duration of coughing, and improve lung imaging findings (*6*).

Tetracyclines such as doxycycline, minocycline, and tetracycline are well-known antibiotics in clinical use. Tetracyclines are known to inhibit matrix metalloproteinases (MMPs) through their ability to chelate zinc compounds on MMPs. Several functions of the coronavirus, including replication, are associated with the host MMPs complex. Therefore, the zinc-chelating properties of tetracyclines may be efficacious against COVID-19 in humans (*7,8*). Tetracycline was also reported to inhibit the binding of the SARS-CoV-2 spike protein to angiotensin-converting enzyme 2 (ACE2) (*9*). Doxycycline inhibits the entry and replication of SARS-CoV-2 *in vitro* (*10*). Yates *et al*. reported that four high-risk patients with COVID-19

and comorbid pulmonary disease were successfully treated with doxycycline, the doses and durations of which were 100-200 mg/day and 5-14 days, respectively (*11*). Meyboy *et al*. reported that treatment of COVID-19 with doxycycline (100 mg, *b.i.d*., for 7 days) alleviated shortness of breath, coughing, a fever, and O_2 saturation (*12*).

Macrolides such as erythromycin, clarithromycin, and azithromycin exhibit antibacterial activity, immunomodulatory action, and anti-inflammatory action. Recently, the antiviral action of macrolides has attracted considerable attention (*13*). Azithromycin accumulates within lysosomes, increases their pH, and hampers lysosomal functions allowing viral replication (*13*). In addition, azithromycin blocks the interaction points between SARS-CoV-2 and the ACE2 receptor, preventing SARS-CoV-2 from invading host cells (*13*). Tsiakos *et al*. reported that treatment with clarithromycin (500 mg, *b.i.d*., for 7 days) was associated with early clinical improvement in patients with moderate COVID-19 (*14*). Moreover, Ghiasvand *et al*. reported that three patients diagnosed with COVID-19 who did not respond to initial treatment improved after additional treatment with azithromycin (*15*).

Apart from the aforementioned macrolide antibiotics, ivermectin, a macrolide antiparasitic agent, is also an inhibitor of SARS-CoV-2, with a single treatment causing a ~5,000-fold reduction in the virus at 48 h in cell culture (*16*). The mechanism by which ivermectin inhibits SARS-CoV-2 is thought to be *via* the inhibition of the nuclear import of viral and host proteins. In specific terms, importin (IMP) α/β1, a host protein, is a heterodimer that binds to the SARS-CoV-2 cargo protein and moves it into the nucleus, where the complex falls apart and the viral cargo can reduce the host cell's antiviral response. Ivermectin destabilizes the IMPα/β1 heterodimer, preventing it from binding to viral protein and thus from entering the nucleus. As a result, the inhibition of antiviral responses is likely to be reduced, leading to a normal, more efficient antiviral response (*16*). Ivermectin also inhibits the binding of the SARS-CoV-2 spike protein to ACE2, much like macrolide antibiotics (*17*). Ahmed *et al*. reported that a 5-day course of ivermectin (12 mg, daily) for COVID-19 reduced the duration of the illness (*18*).

Examining multidrug therapy for COVID-19, Gautret *et al*. reported that a combination of hydroxychloroquine (200 mg, *t.i.d*., for 10 days) and azithromycin (500 mg on day 1, followed by 250 mg, daily, for the next 4 days) reduced the viral load to an undetectable level on day 6. Moreover, this combined therapy proved to be superior to hydroxychloroquine monotherapy (*19*). Alam *et al*. reported that a combination of ivermectin (0.2 mg/kg, single dose) and doxycycline (100 mg, daily, for 10 days) was efficacious in viral clearance in patients with mild or moderate COVID-19 (*20*). Procter *et al*. treated outpatients with COVID-19 with at least two agents with antiviral activity against SARS-CoV-2 (zinc, hydroxychloroquine, and ivermectin) and one antibiotic (azithromycin, doxycycline, and ceftriaxone) along with inhaled budesonide and/or intramuscular dexamethasone. Consequently, multidrug therapy of early ambulatory patients (not hospitalized and treated at home) was found to be safe, feasible, and associated with low rates of hospitalization and mortality (*21*). Prasad reported a patient with COVID-19 and pulmonary lesions who recovered after receiving early treatment with ivermectin (6 mg, *b.i.d.*, for 3 days), azithromycin (500 mg, daily, for 5 days), doxycycline (100 mg, *b.i.d.*, for 5 days), and prednisolone (50 mg, daily, for 5 days) followed by dexamethasone (6 mg, daily) (*22*). Apart from the aforementioned multidrug therapy, a combination of ivermectin and azithromycin (*23*), a combination of doxycycline and azithromycin (*24*), and a combination of hydroxychloroquine, ivermectin, and azithromycin (*25*) are proposed treatments to be studied in clinical trials.

Multidrug therapy is more efficacious than single-drug therapy because there are differences in the mechanisms of action of the drugs described. Moreover, multidrug therapy may prevent the emergence of drug-resistant SARS-CoV-2. Clinical trials need to be conducted to better assess the efficacy and tolerability of the aforementioned multidrug therapy before it can be adopted on a wider basis.

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COVID-19 in patients living with human immunodeficiency virus (HIV) infection: Challenges and way-forward

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SUMMARY Most studies have described worse outcomes with coronavirus disease 2019 (COVID-19) in patients with human immunodeficiency virus (HIV). This has been attributed to COVID-19 associated lymphopenia (resulting in lower CD4 count), higher prevalence of comorbidities (established risk factors for severity in COVID-19) and pre-existing lung damage. The problem has been further aggravated by the lack in the access to routine care in HIV patients due to diversion of resources. In this article, we discuss the impact of COVID-19 on patients with HIV infection.

Keywords COVID-19, HIV, tuberculosis

The clinical spectrum of coronavirus disease 2019 (COVID-19) is diverse and ranges from asymptomatic infection to a life-threatening illness. The severity of COVID-19 has been shown to be significantly associated with diabetes, hypertension, and cardiovascular disease (*1*). It is worthwhile to note that some of these comorbidities are more frequently present in patients living with human immunodeficiency virus infection (PLHIV). COVID-19 is associated with lymphopenia, and therefore, an absolute number of CD4 lymphocytes is also expected to decrease in these patients. Hence, infection with COVID-19 can theoretically reduce CD4 count in PLHIV. A decrease in CD4 count is associated with increased susceptibility to opportunistic infections (*2*). Also, PLHIV may have pre-existing lung damage due to history of opportunistic infections such as tuberculosis. A new COVID-19 infection in these patients with pre-existing lung damage can be associated with higher severity (*3,4*).

Initial studies suggested no increase in adverse outcomes in PLHIV with COVID-19 when compared to COVID-19 patients without human immunodeficiency virus (HIV) infection (*5,6*). However, in a large study from South Africa, poor outcomes were noted in PLHIV with COVID-19 (7). Another study with a large sample size showed worse COVID-19 related outcomes in patients with HIV (*8*). Lower CD4 count was found to be a significant risk factor of mortality in that study (*8*).

The immunosuppression associated with HIV may interfere with antibody response against COVID-19 (*9*). This may have potential implication in surveillance and vaccine effectiveness. Protease inhibitors like lopinavir/ritonavir used in the treatment of HIV were initially suggested to have a beneficial impact on COVID-19 infection. However, published trials have later refuted the role of lopinavir/ritonavir in improving the disease progression and outcomes (*10*).

The economic resources and manpower for HIV care have been shunted to COVID-19 centres. The lockdown imposed at various times has inadvertently affected the access to HIV testing and care. This, coupled with the loss of daily wages of PLHIV or their caretakers, has further added fuel to the problem. Regular psychosocial counselling and public awareness campaigns held at HIV clinics for mitigating the social stigma has also come to a halt. This may have contributed adversely towards the mental well-being of PLHIV.

Ensuring adequacy of drug supplies at the HIV centres and involvement of local/regional stakeholders for the delivery of the drug is paramount. It is vital to ensure the follow-up of PLHIV in quarantine or isolation to check their adherence to medications. They should also be monitored for drug toxicities and complications. Use of telemedicine (locally used mobile applications/ messaging services) will also be helpful to ensure retention in care. There is a need to facilitate the continuum of health care delivery services to PLHIV during the COVID-19 pandemic.

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Novel anticancer drugs approved in 2020

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SUMMARY Cancer is still a major factor threatening human life around the world, and anticancer drugs remain a huge unmet clinical need. Here, we reviewed novel drugs including new molecular entities and new therapeutic biologics approved in the US, EU, Japan, and China that represent the main advances in anticancer drug research and development in 2020. Small molecule inhibitors targeting oncogenes, antibodies, and antibody drug conjugates (ADCs) are the main anticancer drugs that were approved in 2020. More novel anticancer drugs that possess target activity and that overcome drug resistance are anticipated in the future.

Keywords anticancer, drugs, US, EU, Japan, China

There were approximately 19.3 million new cases of cancer around the world in 2020, and approximately 10 million people died of that disease that year according to the Global Cancer Statistics Report (*1*). An important therapeutic strategy, anticancer drugs remain a huge unmet clinical need in this era. Here, we review novel anticancer drugs including new molecular entities and new therapeutic biologics that were approved in the US, EU, Japan, and China in 2020 (Table 1).

Lung cancer is the second most common cancer and the leading cause of death from cancer (2.2 million cases, 1.8 million deaths) worldwide (*1*). There are two main subtypes of lung cancer, small-cell lung cancer (SCLC) and non-small-cell lung cancer (NSCLC), that account for about 76% of all lung cancers (*2*). In the past year, the US Food and Drug Administration (FDA) has approved three anti-NSCLC drugs including selpercatinib, pralsetinib, and capmatinib (Table 1). Selpercatinib and pralsetinib target the RET (rearranged during transfection). Selpercatinib was approved for treatment of RET fusion-positive NSCLC that has spread in adults (*3*). It was also approved for treatment of advanced or metastatic medullary thyroid cancer and advanced RET fusion-positive thyroid cancer in patients age 12 and older who require systemic therapy (*3*). Pralsetinib was approved for adult patients who suffer from metastatic RET fusion-positive NSCLC (*4*). Capmatinib is a kinase inhibitor that targets the MET (mesenchymal-epithelial transition) (*5*). Capmatinib is mainly used for adults with locally advanced or metastatic non-small cell lung cancer whose tumors have a mutation that leads to mesenchymal-epithelial transition (MET) exon 14 skipping (METex14) (*5*). Japan approved another MET inhibitor, tepotinib, for the treatment of patients with unresectable, advanced, or recurrent NSCLC with METex14 (*6*). Tepotinib was first approved in Japan in 2020 as a "line-agnostic" drug, which means that the drug has been approved for patients who have not received treatment and patients who failed to respond to previous treatment. In 2020, the National Medical Products Administration (NMPA) of China approved two anti-NSCLC drugs: almonertinib and ensartinib. Almonertinib, a third-generation epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, was approved for adult patients with locally advanced or metastatic NSCLC who have been treated with EGFR-TKI and who have a T790M mutation (*7*). Ensartinib is indicated for anaplastic lymphoma kinase (ALK)-positive locally advanced or metastatic NSCLC that has progressed after patients received crizotinib therapy or who developed a tolerance to crizotinib (*8*). NMPA approved ensartinib as a second-generation ALK inhibitor. Although SCLC accounts for a small percentage of lung cancer, it has a shorter doubling time, higher growth fraction, and earlier development of metastases compared to NSCLC. Lurbinectedin, a cytotoxic drug approved in the US, is used to treat adult patients with metastatic SCLC whose disease has progressed during or after platinum-based chemotherapy (*9*).

Breast cancer (2.3 million cases) surpassed lung cancer (2.2 million cases) as the most common type of cancer worldwide in 2020 (*1*). Four anti-breast cancer drugs have been approved by the US and China, including 3 drugs targeting human epidermal growth factor receptor 2 (HER2) - inetetamab, margetuximab, and tucatinib - and 1 antibody-drug conjugate (ADC),

Table 1. Approved anticancer drugs in the US, EU, Japan, and China in 2020

Drug	Target	Indication	Country/District	*Ref.*
Selpercatinib	RET	Non-small cell lung cancer, thyroid cancer	U.S.	(*3*)
Pralsetinib	RET	Non-small cell lung cancer	U.S.	(*4*)
Capmatinib	MET	Non-small cell lung cancer	U.S./Japan	(*5*)
Tepotinib	MET	Non-small cell lung cancer	Japan	(*6*)
Almonertinib	EGFR	Non-small cell lung cancer	China	(*7*)
Ensartinib	ALK	Non-small cell lung cancer	China	(*8*)
Lurbinectedin	DNA	Small cell lung cancer	U.S.	(*9*)
Inetetamab	HER2	Breast cancer	China	(*10*)
Margetuximab	HER2	Breast cancer	U.S.	(*11*)
Tucatinib	HER2	Breast cancer	U.S.	(*12*)
Sacituzumab govitecan	TROP-2	Breast cancer	U.S.	(*13*)
Cedazuridine/decitabine	Cytidine deaminase/ DNA methyltransferase	Myelodysplastic syndromes and chronic myelomonocytic leukemia	U.S.	(*14*)
Belantamab mafodotin	BCMA	Multiple myeloma	U.S./EU	(*15*)
Isatuximab	CD38	Multiple myeloma	U.S./EU/Japan	(*16*)
Tafasitamab	CD19	Diffuse large B-cell lymphoma	U.S.	(*17*)
Tirabrutinib	BTK	Primary central nervous system lymphoma	Japan	(*18*)
Orelabrutinib	BTK	Mantle-cell lymphoma, chronic lymphocytic leukemia, small lymphocytic lymphoma	China	(*19*)
Ripretinib	KIT and PDGFRA	Gastrointestinal stromal tumor	U.S.	(*20,21*)
Avapritinib	PDGFRA	Gastrointestinal stromal tumor	U.S./EU	(*22*)
Surufatinib	VEGFR and FGFR1	Neuroendocrine tumors	China	(*23*)
Naxitamab	GD2	Neuroblastoma	U.S.	(*24*)
Pemigatinib	FGFR2	Cholangiocarcinoma	U.S.	(*25*)
Cetuximab saratolacan	EGFR	Head and neck cancer	Japan	(*26*)
fluzoparib	PARP	Ovarian cancer, fallopian tube cancer, or primary peritoneal cancer	China	(*27*)
Tazemetostat	EZH2	Epithelioid sarcoma	U.S.	(*28*)

sacituzumab govitecan, which targets tumor-associated calcium signal transducer 2 (TROP-2) (Table 1). Inetetamab combined with vinorelbine was approved in China for patients with HER2-positive metastatic breast cancer who have received one or more chemotherapy regimens (*10*). In the US, margetuximab combined with chemotherapy is indicated for treatment of adults with metastatic HER2-positive breast cancer who have received two or more anti-HER2 regimens, at least one of which is used for metastatic disease (*11*). Tucatinib in combination with trastuzumab and capecitabine was approved in the US to treat patients with advanced unresectable or metastatic HER2-positive breast cancer who have received one or more prior anti-HER2-based regimens (*12*). Sacituzumab govitecan, approved in the US, is a TROP-2-directed antibody and topoisomerase inhibitor drug conjugate for the treatment of adult patients with metastatic triple-negative breast cancer (TNBC) who have previously received at least 2 therapies (*13*).

Six new drugs to treat hematopoietic malignancies, including cedazuridine/decitabine, belantamab mafodotin, isatuximab, tafasitamab, tirabrutinib and orelabrutinib (Table 1), were approved in the US, EU, Japan, and China in 2020. Decitabine/cedazuridine, which suppresses cytidine deaminase and DNA methyltransferase respectively, was approved as a fixed-dose combination medication for adults with myelodysplastic syndromes (MDS) and chronic myelomonocytic leukemia (CMML) (*14*). Belantamab mafodotin and isatuximab are indicated for treatment of multiple myeloma. Belantamab mafodotin, a humanized monoclonal antibody against the B-cell maturation antigen (BCMA) conjugated with the cytotoxic agent maleimidocaproyl monomethyl auristatin F, was approved in the US and EU in 2020 for patients with elapsed or refractory multiple myeloma who have previously received at least four treatments, including an anti-CD38 monoclonal antibody, a proteasome inhibitor, and an immunomodulator (*15*). A CD38-directed cytolytic antibody, isatuximab was approved in the US, EU, and Japan in 2020 for use in combination with pomalidomide and dexamethasone for adult patients with multiple myeloma who have received at least two prior therapies including lenalidomide and a proteasome inhibitor (*16*). Tafasitamab, tirabrutinib, and orelabrutinib are indicated for treatment of lymphoma. Tafasitamab, a humanized Fc-modified cytolytic CD19 antibody, was approved in the US in combination with lenalidomide, for treatment of adult patients with relapsed/refractory diffuse large B-cell lymphoma (DLBCL) (*17*). Both tirabrutinib and orelabrutinib are Bruton's tyrosine kinase (BTK) inhibitors. In Japan, tirabrutinib was approved to treat relapsed or refractory primary central nervous system lymphoma (PCNSL) (18). In China, orelabrutinib was approved for treatment of patients with adult mantle cell lymphoma (MCL) or adult chronic lymphocytic leukemia (CLL)/small

lymphocytic lymphoma (SLL) who have received at least one prior treatment (*19*).

Other new anticancer drugs approved in the past year include ripretinib for gastrointestinal stromal tumors (GISTs), avapritinib for GISTs, surufatinib for neuroendocrine tumors, naxitamab for neuroblastoma, pemigatinib for cholangiocarcinoma, cetuximab saratolacan for head and neck cancer, fluzoparib for ovarian cancer, and tazemetostat for epithelioid sarcoma. Ripretinib, a kinase inhibitor suppressing KIT and platelet-derived growth factor receptor A (PDGFRA), was approved in the US for treatment of adults with advanced GIST who have received prior three or more kinase inhibitor therapies, including imatinib (*20,21*). Avapritinib, approved in both the US and EU, is also a PDGFRA inhibitor that is indicated for treatment of adults with unresectable or metastatic GIST harboring the PDGFRA D842V mutation (*22*). Surufatinib targets the vascular endothelial growth factor receptor (VEGFR) and fibroblast growth factor receptor 1 (FGFR1) and was approved in China as a single agent for non-pancreatic and well differentiated neuroendocrine tumors that cannot be surgically removed or are metastatic (*23*). Naxitamab combined with granulocyte-macrophage colony stimulating factor (GM-CSF) was approved in the US and is indicated for treatment of patients 1 year of age and older with high-risk neuroblastoma in bone or bone marrow (*24*). Pemigatinib, an inhibitor of fibroblast growth factor receptor 2 (FGFR2), is used to treat patients with locally advanced or metastatic cholangiocarcinoma with an FGFR2 fusion or rearrangement who have received previous treatment (*25*). Cetuximab saratolacan, a chemical conjugate of the photosensitizer IR700 with cetuximab, targets EGFR and was approved in Japan to treat unresectable locally advanced or recurrent head and neck cancer (*26*). Fluzoparib, a small molecule poly(ADP-ribose) polymerase (PARP) inhibitor, was approved in China to treat patients with platinum-sensitive recurrent ovarian cancer, fallopian tube cancer, or primary peritoneal cancer with a germline BRCA mutation (gBRCAm) who have undergone second-line or later chemotherapy (*27*) (Table 1). Tazemetostat, an inhibitor of enhancer of zeste homolog 2 (EZH2), is indicated for treatment of adults and adolescents age ≥ 16 years with metastatic/locally advanced epithelioid sarcoma that is not eligible for complete resection (*28*).

Although the world struggled with COVID-19 in 2020, significant progress has nonetheless been made in cancer drug research. Small molecule inhibitors targeting oncogenes, antibodies, and antibody drug conjugates (ADCs) were the main anticancer drugs approved in 2020. More novel anticancer drugs that possess target activity and that overcome drug resistance are anticipated in the future.

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Letter

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Visceral leishmaniasis masquerading as drug-induced pancytopenia in myasthenia gravis

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SUMMARY Visceral leishmaniasis (VL), also known as kala-azar (black fever in Hindi), is a disease primarily caused by *Leishmania donovani*. The most important clinical manifestation of visceral leishmaniasis is fever. Nonspecific laboratory findings of visceral leishmaniasis include anemia, neutropenia, eosinopenia, and thrombocytopenia. Definitive diagnosis of visceral leishmaniasis requires the demonstration of either parasite by smear or tissue by culture (usually bone marrow or spleen). Myasthenia gravis is an autoimmune disease caused by antibodies to acetylcholine receptors in the post-junctional membrane of the neuromuscular junction. It typically presents with fatigable muscle weakness without any sensory or brain involvement. It is usually treated with corticosteroids and immunosuppressants like azathioprine. Here we encountered a confirmed case of myasthenia gravis on azathioprine with pancytopenia. While working up to evaluate pancytopenia, bone marrow examination revealed presence of Donovan bodies and the patient showed good response to liposomal amphotericin-B. In retrospect, a case of myasthenia gravis, who presented with pancytopenia presumably drug-induced, was found to have visceral leishmaniasis.

Keywords Immunosuppression, myasthenia gravis, pancytopenia, visceral leishmaniasis

Visceral leishmaniasis, or kala azar, is an extremely rare condition encountered in the urban population of West Bengal, India. Also, its prevalence is very low among non-HIV-infected patients who are on corticosteroids or other immunosuppressive therapies. There is limited data on the risk factors for developing visceral leishmaniasis in persons on immunosuppressive treatment. Herein we describe a patient of myasthenia gravis on chronic immunosuppressive therapy suspected with azathioprine-induced pancytopenia but eventually diagnosed to have visceral leishmaniasis.

A 62-year-old gentleman, a resident of Kolkata, initially presented to the neurologist with ptosis & limb weakness and was diagnosed to have generalized myasthenia gravis. Repetitive nerve stimulation test was positive. He was started on pyridostigmine (60 mg every 6 hourly) and prednisolone therapy (starting dose 1 mg/kg/day). Subsequently, azathioprine was added as a steroid-sparing agent, and prednisolone dose was tapered to 10 mg/day. After about a year of azathioprine therapy, he developed pancytopenia, but there was no fever or bleeding manifestations, and his myasthenic symptoms were well-controlled. There was no past history of blood transfusion or travel to kala-azar endemic districts. Azathioprine was stopped and bone marrow aspiration and biopsy was performed given the persistent pancytopenia (hemoglobin 6.6 gm/dl, MCV 111 fL, MCH 33.2 pg, total leucocyte count of 2,200/μL with 56% neutrophils, 33% lymphocytes, 11% monocytes, and platelet count 86,000/μL). Clinical examination revealed pallor, mild hepatomegaly (2 cm below costal margin), and moderate splenomegaly (4 cm below costal margin), but there was no peripheral lymphadenopathy, sternal tenderness or skin lesions. Renal and liver function tests, blood glucose, electrolytes, serum ferritin, iron, vitamin B12 and folate levels were normal; viral serology for HIV, HBsAg, anti-HCV were non-reactive. Bone marrow aspiration cytology and biopsy revealed normal trilineage hematopoiesis, adequate marrow storage iron along with numerous intracellular and extracellular Leishman-Donovan (L-D) bodies, thereby confirming the diagnosis of visceral leishmaniasis (Figure 1). The patient was treated with liposomal amphotericin-B (total cumulative dose of 21 mg/kg

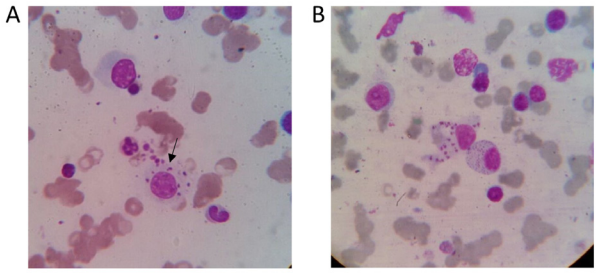

Figure 1. (A) Romanowsky stain showing intracellular LD bodies within macrophage in High power view (40×) (A) and Oil immersion view (100×) (B): marked with arrow.

body weight) and showed complete clinical response. Treatment for myasthenia was subsequently resumed, and he is doing well on follow up.

Leishmaniasis refers to a diverse spectrum of clinical syndrome caused by infection with a protozoan parasite of genus *Leishmania* acquired by the bite of a sandfly (*1*). The most common presentation of visceral leishmaniasis is the abrupt onset of moderate to high-grade fever associated with rigors and chills. Skin gradually develops dark discoloration due to hyperpigmentation (*2*). Except in the Indian subcontinent and Africa, where all age groups are affected, it is a disease of infants and small children.

The number of leishmaniasis cases associated with immunosuppression has increased regularly over the past 20 years. Immunosuppression related to human immunodeficiency virus (HIV) infection, immunosuppressive treatment, organ transplantation, and neoplastic diseases increases the risk for *Leishmania*-infected people to develop visceral illness (*3,4*). Immunosuppression is one of the most substantial risk factors for overt clinical disease, and can also alter disease presentation and treatment response. Although immunosuppression has been mainly observed in HIV-infected patients, non-HIV related immunosuppressive conditions are becoming increasingly prevalent globally, mainly because of better medical care of patients with chronic illnesses and the therapeutic use of immunosuppressive drugs. Visceral leishmaniasis has also been reportedly associated with the use of various immunosuppressive drugs, such as azathioprine, methotrexate, steroids, cyclosporine, and cyclophosphamide (5).

The fact that immunosuppressive conditions pose a real challenge in visceral leishmaniasis endemic regions is illustrated by a *Leishmania* community outbreak in Madrid (*6*). Among the 446 cases detected between July 2009 and December 2012, 15.2% (n = 68) had immunosuppressive conditions, mostly non-HIV-related. Overall, 31.3% of visceral leishmaniasis cases and 6.3% of CL cases were diagnosed in immunosuppressed individuals.

Visceral leishmaniasis is a potentially fatal infection in immunocompromised hosts, and current therapies have failed to eradicate *L. donovani* from infected tissue (*7*). In such conditions, unusual forms of leishmaniasis can develop and foster the risk of a fatal diagnostic delay and of a poor response to therapy (*8*).

Our patient did not give any history of travel to endemic areas with distribution of sand-fly vectors. He did not have fever or skin pigmentation as the initial presentation; neither was he associated with any immunosuppressive condition. He presented with pancytopenia while being treated for myasthenia with oral corticosteroids and azathioprine as a steroid-sparing agent. Though pancytopenia is a crucial feature and occurs early in the course of visceral leishmaniasis, it was not considered as a presenting feature in the index case because of the absence of fever and epidemiological factors. Rather a bone marrow aspiration and biopsy was contemplated which clinched the diagnosis of leishmaniasis and definitive treatment was offered.

Silvia et al. had reported a case where prolonged steroid use was likely to be associated to the clinical severity of the disease (*9*). In the female patient affected by myasthenia, the relapses, the clinical spread to the gastrointestinal tract, and the severe T lymphocyte defects were all factors likely to be related to the sustained impairment of the immune response.

Nevertheless, unusual presentations of leishmaniasis have to be suspected as a differential diagnosis in patients with immunosuppressive conditions other than HIV infection. In such patients, the occurrence of lymphopenia, anemia, pancytopenia or hypergammaglobulinemia even in the absence of fever or a positive travel history, particularly in kala azar-endemic countries should alert clinicians to include leishmaniasis in their differential diagnosis.

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Policy Forum articles discuss research and policy issues in areas related to life science such as public health, the medical care system, and social science and may address governmental issues at district, national, and international levels of discourse. Policy Forum articles should not exceed 3,000 words in length (excluding references) and should be limited to a maximum of 5 figures and/or tables and 30 references.

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Communications are short, timely pieces that spotlight new research findings or policy issues of interest to the field of global health and medical practice that are of immediate importance. Depending on their content, Communications will be published as "Comments" or "Correspondence". Communications should not exceed 1,500 words in length (excluding references) and should be limited to a maximum of 2 figures and/or tables and 20 references.

Editorials are short, invited opinion pieces that discuss an issue of immediate importance to the fields of global health, medical practice, and basic science oriented for clinical application. Editorials should not exceed 1,000 words in length (excluding references) and should be limited to a maximum of 10 references. Editorials may contain one figure or table.

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

Letters should present considered opinions in response to articles published in *Drug Discoveries & Therapeutics* in the last 6 months or issues of general interest. Letters should not exceed 800 words in length and may contain a maximum of 10 references. Letters may contain one figure or table.

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Manuscripts are suggested to be prepared in accordance with the "Recommendations for the Conduct, Reporting, Editing, and Publication of Scholarly Work in Medical Journals", as presented at *http://www.ICMJE.org*.

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Examples are given below:

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Nakata M, Tang W. Japan-China Joint Medical Workshop on Drug Discoveries and Therapeutics 2008: The need of Asian pharmaceutical researchers' cooperation. Drug Discov Ther. 2008; 2:262-263.

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Darby S, Hill D, Auvinen A, *et al*. Radon in homes and risk of lung cancer: Collaborative analysis of individual data from 13 European case-control studies. BMJ. 2005; 330:223.

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

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