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Preserved SARS-CoV-2 neutralizing IgG activity of in-house manufactured COVID-19 convalescent plasma

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ABSTRACT

Purpose: In the current study, we aimed to evaluate the neutralizing IgG activity against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) as well as the coagulation factors of convalescent plasmas which we manufactured in-house without a fast-freezing technique.
Methods: We collected plasmas from eligible participants who had confirmed certain titers of neutralizing antibodies. The plasmas were frozen and stored in the ordinary biofreezer without a fast-freezing function. The purified-IgG neutralizing activity of 20 samples from 19 participants and the coagulation factors of 49 samples from 40 participants were evaluated before and after freezing.
Results: Purified-IgG maintained its neutralizing activities, with the median 50 % inhibitory concentration (IC50) of 10.11 mg/ml (IQR 6.53–18.19) before freezing and 8.90 m g/ml (IQR 6.92–28.27) after thawing ($p = 0.956$). On the contrary, fibrinogen and factor Ⅷ decreased significantly after freezing and thawing in our environment. No significant temperature deviation was observed during the storage period.
Conclusion: Neutralizing IgG activity, which largely contributes to the antiviral activity of convalescent plasma, did not change through our in-house manufacturing, without fastfreezing and storage conditions for more than 200 days. Ordinary freezers without the fast-freezing function are suitable enough to manufacture and store convalescent plasmas. Hospitals or facilities without specified resources could easily collect and store convalescent plasmas in case of upcoming emerging or re-emerging infectious diseases on-demand with appropriate neutralizing antibody levels measurements.

1. Introduction

Convalescent plasma has been used as passive immunotherapy for the prevention and treatment of various infectious diseases historically [1]. Convalescent plasma has recently emerged as an effective treatment option for diseases such as Ebola virus disease, Severe Acute Respiratory Syndrome (SARS), Middle East Respiratory Syndrome (MERS), or maybe future emerging pathogens. At the end of 2019, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection, which causes severe cases of pneumonia (COVID-19), was reported in Wuhan, Hubei Province, China, which proceeded to spread worldwide [2]. There were few standard therapies with strong evidence early in the pandemic; thus, several drugs were proposed for compassionate or experimental use, such as remdesivir, hydroxychloroquine with or without azithromycin,

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lopinavir/ ritonavir, or ivermectin. Moreover, convalescent plasma was a promising candidate for therapeutic drug for COVID-19, and several randomized clinical trials have been done to test this [3].

In Japan, all blood products are manufactured from voluntary healthy donors. The in-house manufacturing of allogenic blood products is restricted except in emergency cases or stem cell transplantation, by the government. The process of manufacturing blood products has already been established for healthy donors. Particularly, all steps of blood donation, checking infections such as hepatitis virus and HIV, freezing, storage, and distribution have been standardized and done safely by the Japan Red Cross Society (JRC), which is the only blood center in Japan. However, at the initiation of the SARS-CoV-2 pandemic, JRC could not accommodate blood donors who recovered from COVID-19 recently because the infectivity of such donors was uncertain. Therefore, our hospital decided to gather convalescent plasma instead of JRC for clinical use. Since we have few experiences in large-scale in-house manufacturing of blood products, we conducted convalescent plasma manufacturing for COVID-19 in collaboration with JRC in April 2020 [4].

To manufacture convalescent plasma products, we refer to the process of fresh frozen plasma (FFP), which is also a plasma product commonly used in hospitals. According to the guidelines for the blood transfusion system, the plasma should be separated as soon as possible after venipuncture and rapidly frozen (so-called fast-freezing) for greater yields of coagulation factor [5,6].

A few experimental reports have showed the superiority of fast-freezing for maintaining coagulation factor VIII activity by comparing contact shock freezers or air blast freezers with just − 40 °C storage [7,8]. Coagulation factor VIII is labile and easily loses activity with the freezing method. However, our hospital does not have such special freezers, and we manufactured convalescent plasma without fast-freezing. We also measured fibrinogen levels as a quality-control measure for manufacture and storage, because fibrinogen is known to be stable through the freeze-thaw cycle regardless of freezing method, but its activity decreases in unfavorable conditions [9].

Convalescent plasma therapy does not aim for replenishment with the coagulation factors but passive immunotherapy by giving anti-SARS-CoV-2 neutralizing antibodies. Immunoglobulins are in general stable for freezing and thawing [10], and convalescent plasma does not seem to need fast-freezing for maintaining its activity. However, there are few reports addressing whether neutralizing activities would be preserved in convalescent plasma without fast-freezing.

Thus, in this study, we aimed to report the quality control of in-house manufactured convalescent plasma. Furthermore, we verified the stability of neutralizing activity of the COVID-19 convalescent plasma product as well as coagulation factors manufactured without fast-freezing.

2. Materials and methods

2.1. Plasma donor recruitment

The inclusion and exclusion criteria were described in our previous report [4]. In brief, individuals aged from 20 to 69 years who had confirmed SARS-CoV-2 infection more than three weeks before the eligibility assessment were enrolled. These individuals were pre-screened without the infections and with the purified IgG viral neutralizing test and/or the amount of anti-SARS-CoV-2 Spike-binding IgG and other hematological and cardiovascular tests. The convalescent plasmas were donated from participants who met the criteria with a certain neutralizing activity or the amount of anti-SARS-CoV-2 Spike-antibodies as previously described with written consent [4].

2.2. Plasma collection and freezing storage

In total, 200–400 ml of plasma was collected from the eligible convalescent donor and distributed into 200–100 ml with a segment for inspection. The donated plasma was frozen and stored in two biofreezers (MDF-U731M-PJ, PHC Holdings Corporation, Tokyo, Japan) at below − 20 °C without fast-freezing. The freezer temperature was monitored and recorded by using two devices: every 5 min with a thermal logger (KR2S, CHINO corporation, Tokyo, Japan) and every 15 min with a thermal logger Temptale Ultra Single use (Sensitech Inc., MA, USA).

Before freezing, the coagulation factors were measured with a segment sample, and neutralizing activity was measured with donated plasma aliquot. After thawing, coagulation factor and neutralizing activity were measured with a segment sample. Frozen plasma was thawed at 4 °C overnight.

2.3. Measurement

Forty-nine samples from 40 participants and 40 samples from 19 participants were evaluated for coagulation analysis and neutralizing antibody analysis, respectively, before freezing and after thawing. Several samples were drawn from identical participants repetitively on other days.

The amount of fibrinogen and the activity of factor VIII were measured at a commercial laboratory (Bio Medical Laboratory, Tokyo, Japan).

Neutralizing antibody activity was measured by the National Center for Global Health and Medicine Research Institute (NCGM-RI), as reported previously [11]. In short, IgG fractions were purified from convalescent plasma by using a Spin column-based Antibody Purification Kit (Protein G) (Cosmo Bio, Tokyo, Japan). The mixture of purified-IgG and the wild-type SARS-CoV-$2^{05-2\ N}$ (PANGO lineage B) were preincubated for 20 min at 37 °C and inoculated to the TMPRSS2-overexpressing VeroE6 cells maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml kanamycin, and 1 mg/ml G418. After culturing the cells for 3 days, the levels of cytopathic effect observed in SARS-CoV-2-exposed cells were determined using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). The purified IgG concentration resulting in 50 % inhibition of the cytopathic effect was defined as a 50 % inhibition concentration (IC_{50}).

2.4. Statistical analysis

Our primary outcome was the alteration of neutralizing activity before and after general freezing and storage. Wilcoxon signed-rank test evaluated the neutralizing activity, the value of fibrinogen, and factor VIII activity. The statistical analysis was done using R software version 4.2.0.

2.5. Ethical approval

This study was approved by the ethics committee of the National Center for Global Health and Medicine (NCGM) (approval no: NCGM-G-003536–08) and was conducted following the Declaration of Helsinki. Written informed consent was obtained from the participants.

3. Results

The characteristics of the specimens are shown in Table 1. We used twenty specimens from nineteen participants and forty-nine specimens from forty participants for neutralizing antibody analysis and coagulation analysis, respectively. The plasmas which were used for neutralizing activity evaluation were stored significantly longer than that used for the coagulation factors evaluation ($p < 0.001$).

We evaluated twenty plasma specimens for the difference in neutralizing activity before and after freezing and thawing (Fig. 1). Before freezing the median IC_{50} value was 10.11 μg/ml (IQR 6.53 – 18.2), and after thawing it was 8.90 μg/ml (IQR 6.92–28.27) ($p =$

Table 1
Participants characteristics.

	Coagulation analysis	Antibody analysis	p value
Number of specimens	49	20	
Number of participants	41	19	
Median age years(range)	54.0 (42.0–63.0)	48.5 (44.3–60.0)	0.258
man (%)	33(67.3 %)	9(45.0 %)	0.146
Median storage days (range)	212 (193–238)	253 (223–311)	< 0.001

0.956).

Both the amount of fibrinogen and the activity of factor VIII significantly decreased after slow-freezing and thawing (Fig. 2). The median fibrinogen levels changed from 241 mg/dL (IQR 216.0 – 271.5) to 196.0 mg/dL (IQR 171.5–224.5) ($p < 0.001$), and median factor VIII activity changed from 123 % (IQR 95.5–135.2) to 47 % (IQR 33.85–57.25) ($p < 0.001$).

Through the storage period, the thermal logger recorded the median temperature (and IQR) in the two freezers as − 27.4(−27.5 to −27.1) °C and − 28.7 (−28.8 to −28.5) °C respectively, although the temperature sometimes transiently rose whenever the freezers were opened for usage. There seemed to be no thermal increase records that could melt the frozen convalescent plasma product.

4. Discussion

We launched a convalescent plasma project in a standard hospital setting without fast-freezing equipment. We evaluated changes in neutralizing activity against SARS-CoV-2, because the freeze-thaw cycle has an impact on the amount or activity of coagulation factors [7–9]. Herein, we did not measure the amount of total human IgG nor

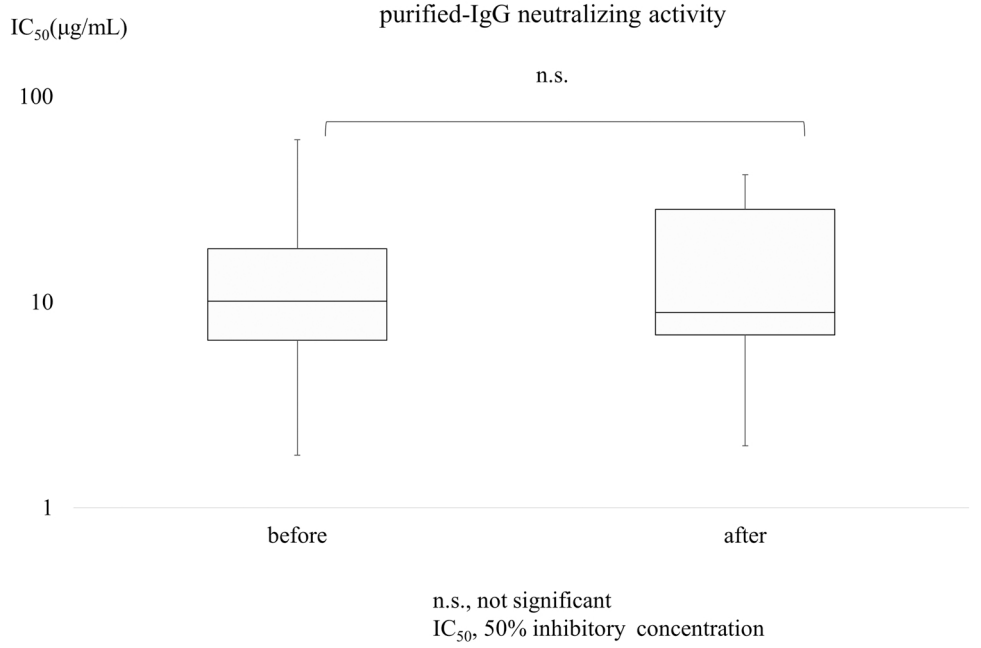

Fig. 1. Comparison of neutralizing activity. Purified-IgG neutralizing activities against SARS-CoV-2 (50 % inhibitory concentration; IC_{50} values) were compared before and after freezing and thawing.

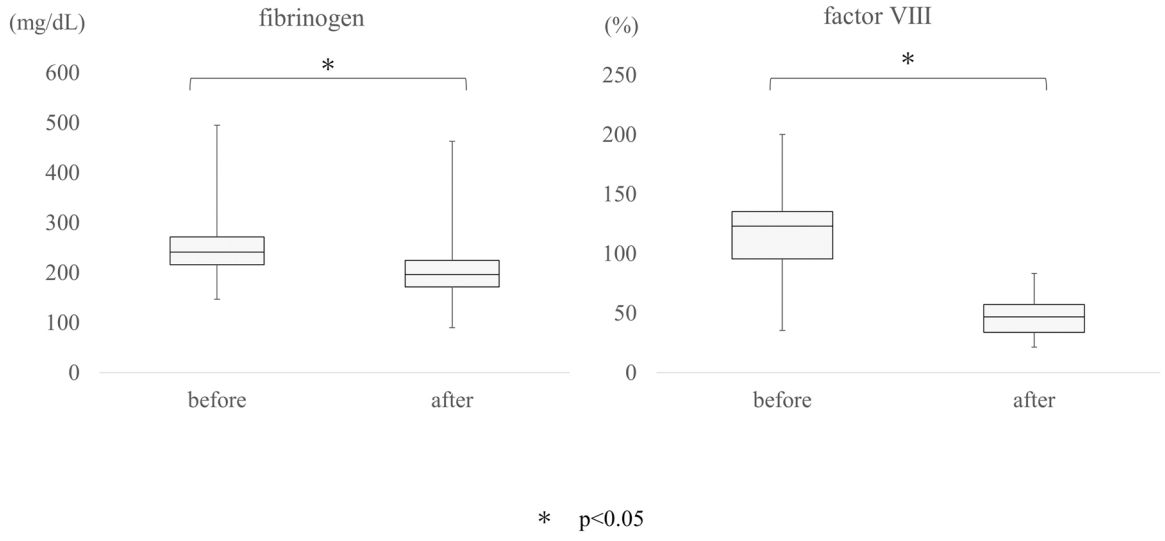

Fig. 2. Comparison of coagulation factors. The amount of fibrinogen and the activity of factor VIII were compared before and after freezing and thawing.

anti-SARS-CoV-2 binding antibodies, because several studies have showed that the freeze-thaw process without fast-freezing had no impact on the immunoglobulin amount [12,13]; thus, we thought that the amount of the neutralizing antibody was preserved regardless of the freezing method. Consequently, the neutralizing antibody activity was found to be preserved, while the amount of fibrinogen and factor VIII activity decreased after the freeze-thaw cycle.

Theoretically, several factors could affect the activity of coagulation factors, including freezing methods, storage temperature and duration, storage portion (main packs or attached segments) and after thawing process. The storage condition was monitored using the thermal logger, and the temperature was maintained below − 20 °C, although transient deviation occurred when the freezer was opened briefly. Storage at − 20 °C for a year did not affect fibrinogen or factor VIII [6], and we do not think the storage condition influenced the coagulation factors.

Factor VIII decreased through the freezing and thawing process, and that could be explained by two reasons. First, we used ordinary bio-freezers without a fast-freezing function, which is required to maintain coagulation factors in manufacturing FFP. Second, we used plasma stored in the segment portion. Coagulation factors in the segment decrease more significantly than those in main packs [14]. Fibrinogen is known to be stable during the freezing process, and our storage conditions seemed adequate. In our study, however, the fibrinogen level decreased after the freeze-thaw cycle owing to unknown reasons. One possible explanation is that frozen plasmas in segments were used for the sample measurement, which could affect the fibrinogen level like factor VIII. Another explanation could be that an accidental temperature change might occur during transportation to the commercial laboratory in a box with dry ice.

We could not clearly provide evidence for the loss in fibrinogen. However, even in such conditions, our in-house plasma maintained its neutralizing activity.

Several studies investigated antibody activity or titer after freeze-thaw cycles, and most of the results described that antibody activity or titer were preserved after the freeze-thaw process [10,15]. Plasma antibodies against SARS-CoV-2 collected from convalescent participants were also evaluated after the freeze-thaw cycle, and the activities did not change significantly [16]. However, to the best of our knowledge, there are no studies evaluating convalescent plasma products manufactured for clinical usage. Our group reported that our convalescent plasmas were safely administered to 11 patients with COVID-19 in a safety study, and only 1 patient had an adverse event (erythema at the infusion site) [17].

The effectiveness of convalescent plasma against COVID-19 has been evaluated in many papers. We recently reported that the administration of highly neutralizing activity confirmed plasma or purified-IgG blocks the disease progression in the Syrian hamster SARS-CoV-2 infectious model [18]. Recent clinical studies demonstrated that early administration of convalescent plasma was effective against COVID-19 among mild to moderate, or severe COVID-19 patients [19,20]. Among the various risk factors, hematological malignancy is known to be associated with severe immunosuppression and high mortality [21], and convalescent plasma therapy is a promising candidate [22]. However, a retrospective study could not show the efficacy of convalescent plasma in COVID-19 patients with concomitant hematological malignancies [23]. In this area, chimeric antigen receptor (CAR) T-cell therapy derived from SARS-CoV-2 convalescent donor is novel potential therapy [24].

Nowadays, there are several treatment options against COVID-19, and the use of the convalescent plasma therapy is controversial. The guideline for the treatment and management of patients with COVID-19 stated supportive comments for convalescent plasma in limited conditions, “among ambulatory patients with mild to moderate COVID-19 at high risk for progression to severe disease who have no other treatment options” [25].

Historically convalescent plasma has been a treatment option for various infectious diseases, and it could be applied to the next emerging or re-emerging infectious diseases [1]. Especially in the early phase of a pandemic, convalescent plasma could be a candidate for treatment options.

We manufactured convalescent plasma products with ordinary freezers and confirmed that this process did not alter the neutralizing antibody activity. Therefore, our study suggests that hospitals could make convalescent plasma products even though they do not have bio-freezers with fast-freezing functions. Since the freezers with fast-freezing functions are very expensive, our results would help many hospitals with limited resources to provide convalescent plasma. To the best of our knowledge, this is the first report of the large-scale in-house manufactured blood products in Japan.

Our study has several limitations. First, investigated specimens had different characteristics, especially storage days. During storage at below − 20 °C, coagulation activity and neutralizing activity may decrease. However, although specimens used for neutralizing activity analysis have a more extended storage period, their neutralizing activity did not show a substantial decrease, unlike coagulation activity. Thus, this difference was unlikely to affect the result. Second, we used several samples collected on other days from some identical donors for the same analysis. Since convalescent plasmas are precious donations and their usage is strictly controlled, we could not use plenty of samples. We used the specimen collected from the same participants at different times to increase the sample number. Third, although coagulation factors could be influenced by the change in temperature, the specimens were shipped to different laboratories (NCGM-RI and BML) and were exposed to different environments during the transportation. Fourth, we did not investigate the in vivo activity in this study. We demonstrated that in vitro neutralizing activity did not decrease during the process. Mainly immunoglobulins are presumed to work as the main component of convalescent plasma. Theoretically, another solubility could be working against infection. Fifth, we could not explain the loss in fibrinogen, as discussed above. The utility of segments as a representation of plasma has limited reports, and further investigation are needed. Finally, we have not compared slow-freezing to fast-freezing directly. Our data only shows that slow-freezing did not alter neutralizing activity (IC_{50}), and whether slow-freezing is equal to fast-freezing is unclear, though presumably, there are no significant differences.

5. Conclusion

We safely collected convalescent plasma from volunteers and manufactured in-house blood products. We then successfully demonstrated that convalescent plasma frozen in ordinary freezers without a fast-freezing function maintained neutralizing activity while coagulation activity decreased during the process. Our findings suggest that fast-freezing is unnecessary for manufacturing convalescent plasma, unlike FFP. Hospitals without fast-freezers or resource-limited countries can make their convalescent plasma product with the usual freezer. It may work against the next emerging or re-emerging infection outbreak.

Compliance with Ethics Guidelines

This study was approved by the ethics committee of the National Center for Global Health and Medicine (NCGM) (approval no: NCGM-G-003536–08) and was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from the participants.

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CRediT authorship contribution statement

Makoto Inada: Conceptualization, Formal analysis, Writing - Original Draft, Writing- Review and editing **Tomiteru Togano:** Conceptualization, Formal analysis, Writing- Review and editing, Supervision **Mari Terada:** Conceptualization, Formal analysis, Data Curation **Katsuyuki Shiratori:** Supervision **Shinya Tsuzuki:** Formal analysis **Yuki Takamatsu:** Methodology, Formal analysis, Writing- Review and editing **Sho Saito:** Supervision Supervision **Akira Hangaishi:** Supervision **Shinichiro Morioka:** Funding acquisition **Satoshi Kutsuna:** Conceptualization, Funding acquisition **Kenji Maeda:** Funding acquisition, Supervision **Hiroaki Mitsuya:** Funding acquisition, Supervision **Norio Ohmagari:** Supervision.

Declaration of interest

None.

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NA.

Authorship

All named authors meet the International Committee of Medical Journal editors (ICMJE) criteria for authorship for this article. They have made substantial contribution to this study, drafted the work or revised it critically for important intellectual content, approved this version to be published, and agreed to be accountable for all aspects of the work.

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