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NOVEL PROTOCOL FOR SELECTION OF SARS-CoV2 CONVALESCENT PLASMA

NOVI PROTOKOL ZA IZBOR DAVAOCA REKOVALENSCENTNE PLAZME SARS-CoV2

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ABSTRACT

Introduction. SARS-CoV2 2019 infection represent global problem. At this moment there is no vaccine or efficient treatment of infected patients. Treatment with blood plasma rich with anti SARS-CoV2 specific antibodies represents rare safe and effective treatment of Covid19 patients.

Patients and Material. A total number of 950 patients were analyzed in this study, whose samples were collected in time interval from 01.05. till 15.08.2020. Patients were enrolled in study from Covid-19 hospitals, out-clinics and as family members of SARS-CoV2 infected patients. Original ELISA tests were developed to measure the concentration of anti-S1S2 Spike and anti-Nucleoprotein (IgG, IgA, IgM) SARS-CoV2 antibodies. Blood convalescent plasma was selectively collected from recovered patients according to specific antibodies concentration.

Results. The highest concentrations of anti-S1S2 Spike or anti-Nucleoprotein specific IgG antibodies were detected in patients with the moderate/heavy clinical form of infection as well as in group of family members of SARS-CoV2 infected patients. Extremely high concentration of anti S1S2 Spike IgG and anti-Nucleoprotein IgG was demonstrated in 3% and 6% (respectively) of patients recovered from severe Covid19. From our hospitalized patients 63% and 51% had modest antibody levels (anti S1S2 Spike and anti-Nucleoprotein, respectively). After 60 days, in our selected donors’ concentration of anti S1S2 Spike IgG antibodies increased in 67%, paralleled with increase of anti-Nucleoprotein IgG antibodies in 58% of donors.

Conclusion. Originally developed ELISA tests enable novel protocol for selection of convalescent blood plasma donor.

Key words: convalescent plasma, SARS-CoV2 specific antibodies.
APSTRAKT

Uvod. Infekcija SARS-CoV2 2019 predstavlja globalni problem. U ovom trenutku ne postoji vakcina niti efikasan tretman zaraženih pacijenata. Lečenje krvnom plazmom bogatom antitelima specifičnim na SARS-CoV2 predstavlja retko sigurno i efikasno lečenje pacijenata sa Covid19.

Pacijenti i materijal. U ovoj studiji analizirano je ukupno 950 pacijenata, čiji su uzorci prikupljeni u vremenskom intervalu od 01.05. do 15.08.2020. Pacijenti su bili uključeni u studije iz bolnica Covid-19, ambulant i kao članovi porodice zaraženih SARS-CoV2 pacijenata. Originalni ELISA testovi su razvijeni za merenje koncentracije antitela na S1S2 Spike i antinukleoproteina (IgG, IgA, IgM) SARS-CoV2. Rekonvalescentna plazma u krvi je selektivno sakupljana od oporavljenih pacijenata prema koncentraciji specifičnih antitela.

Rezultati. Najveće koncentracije antitela na S1S2 Spike ili antitela na Nukleoprotein otkrivene su kod pacijenata sa umerenim / teškim kliničkim oblikom infekcije. Najveća koncentracija anti S1S2 Spike ili anti Nukleoprotein IgG specifičnih antitela detektovana je u pacijenata sa srednje teškom/teškom kliničkom formom infekcije, kao i u članova porodice SARS-CoV2 pacijenata. Ekstremno visoke koncentracije anti S1S2 Spike IgG i anti Nukleoprotein IgG su demonstrirane u 3% i 6% pacijenata oporavljenih od teškog Covid19. Od naših hospitalizovanih pacijenata 63% i 51% su imali minimalne vrednosti antitela (anti S1S2 Spike i anti Nukleoprotein). Nakon 60 dana, u plazmi izabranih donora koncentracija anti S1S2 Spike IgG antitela porasla je za 67% paralelno sa porastom anti-Nukleoprotein IgG antitela u 58% donora.

Zaključak. Originalno razvijeni ELISA testovi omogućavaju novi protokol za odabir davaoca rekonvalescentne krvne plazme.

Ključne reči: rekonvalescentna plazma, SARS-CoV2 specifična antitela.
INTRODUCTION

To date, there are no drugs approved by the US Food and Drug Administration (FDA) for the treatment of patients with COVID-19. Current clinical research includes measures for infection prevention and control and, also, supportive care, including oxygen and mechanical ventilation support when necessary. Myriad of drugs that have been approved for other indications, are used, as well as a variety of new drugs whose effects are being studied in several hundreds of clinical trials that are underway around the world. Scientists around the world are working tirelessly, and information about the mechanisms of transmission, the clinical spectrum of the disease, new diagnostics and strategies for prevention and therapy is spreading rapidly. In general, there are many unknowns regarding the virus-host interaction, the development of the epidemic, the possibilities and success of treatment, and any research in this field is extremely important.

The significance of the corona virus disease pandemic 2019 (COVID-19), due to the new severe acute respiratory syndrome of corona virus 2 (SARS-CoV-2), caused a sudden and significant increase in the number of hospitalizations in the world due to pneumonia with multiorgan disease. SARS-CoV-2 is spread primarily by respiratory droplets - close face-to-face contact. The infection can be spread by asymptomatic, presymptomatic and symptomatic carriers. The average time from exposure to the virus to the onset of symptoms is 5 days, and 97.5% of people develop symptoms within 11.5 days. The most common symptoms are high fever, dry cough and difficulty of breathing. Radiographic and laboratory abnormalities, such as lymphopenia and elevated lactate dehydrogenase, are common but nonspecific. The diagnosis is made by detecting SARS-CoV-2 by PCR (polymerase chain reaction), although false-negative test results can occur in 20% to 67% of patients, depending on the quality and timing of the test. The disease may have an asymptomatic or fulminant course, characterized by sepsis and acute respiratory failure. Approximately 5% of patients with COVID-19, ie. 20% of those hospitalized have serious symptoms that require intensive care. More than 75% of patients hospitalized with COVID-19 require additional oxygen, which includes the best therapeutic measures for the treatment of acute hypoxic respiratory failure (1).
In current trials, antiviral therapy, immune modulators, and anticoagulants are being tested. The death rate from COVID-19 varies significantly depending on age, ranging from 0.3 deaths per 1,000 patients (age 5 to 17 years) to 304.9 deaths per 1,000 cases in patients aged 85 years or older in the United States. Among patients hospitalized in the intensive care unit, the mortality rate is up to 40%. At least 120 SARS-CoV-2 vaccines are being prepared. Until an effective vaccine is available, the primary methods for reducing the spread are face masks, social distancing as well as the application of all therapeutic modalities (1).

Since some 10% of patients fail to cope with this disease despite the applied measures, doctors initiated collecting and therapeutic application of convalescent plasma from recovered COVID-19 patients, after 14 days from the end of the disease. The idea is old, and it is based on the use of plasma rich in IgG antibodies, which would facilitate the fight, support immunity and alleviate the clinical picture. Four to six or eight weeks after infection, there should be enough antibodies in the patient's blood to neutralize the virus and theoretically limit the infection (1).

Plasma donors can be convalescents who have had a confirmed positive PCR test for the virus, no symptoms for 14 days, negative PCR test at the time of donation - and have a high titer of IgG antibodies. In most cases, it is a period of one month (2).

Treatment with plasma from patients recovered from viral infections was first reported during the 1918 influenza pandemic. The first report of 5 critically ill patients with COVID-19 treated with convalescent plasma containing neutralizing antibodies showed an improvement in clinical status in all participants. This was reflected as a combination of changes in body temperature, assessment of organ involvement, oxygen partial pressure, viral load, serum antibody titers, routine blood biochemical index, ARDS (acute respiratory distress syndrome), ventilation and extracorporeal membrane oxygenation monitored before and after administration convalescent plasmas (3).

However, a subsequent multicenter, randomized clinical trial in China of 103 patients with severe COVID-19 found no statistical significance in the time to clinical improvement within 28 days in patients receiving convalescent plasma compared to standard treatment (51.9% compared to 43.1%) (4). Since the study was discontinued, this limited the possibility of detecting a clinically important difference. Alternative approaches include the
use of convalescent hyperimmune globulin produced from plasma and monoclonal antibodies directed to SARS-CoV-2 (5-6).

The virulence of a particular virus is often considered related with the immune response it encounters in the human body. For Covid-19, the immune response is divided into two phases. The initial phase is thought to involve the development of a specific adaptive and immune response needed to eliminate the virus and stop the disease from progressing. It is therefore important to provide treatments that have previously stimulated an immune response, such as antibodies and immunomodulators (2,6); there may be a loss of 20% of its own antibodies, that will resume a few days later. Immune response, however, weakens with age, making the elderly particularly vulnerable. If the immune response is weak or damaged due to other complications such as cardiovascular disease and diabetes, the virus multiplies and can lead to tissue damage. The second phase of the immune response leads to damage of the cells that caused the pneumonia. Such infection is very dangerous, since pneumonia causes respiratory disorders, making it difficult for individuals to breathe on their own. Different therapies are tested in different phases of the disease, making it important to identify exact phase of the patient’s disease, before starting treatment (1).

The World Health Organization warns that there is no evidence that the presence of antibodies means that you are protected from reinfection with Covid-19. The level of immunity and how long the immunity lasts are still unknown. Ongoing studies will eventually reveal more data on this.

Due to all of the above, any research related to Covid-19 therapy is crucial, including the collection and administration of convalescent’s plasma to patients.

Patients with resolved viral infection will develop an immune response with polyclonal antibodies to various CoV-2019 viral antigens, and some of these polyclonal antibodies, if the patient has them in high titers, will probably neutralize the virus and prevent more severe forms of infection. Doctors are trying to use this fact in a therapeutic sense, and that is why the idea of collecting plasma (rich in antibodies) from the convalescents after Covid came about (1-13).

The potential danger of using such plasma in terms of side effects in recipients, including but not limited to allergic reactions, acute lung injury, and circulatory overload in patients with cardiac disorders, must also be mentioned here (14-16).
Our institution also worked on testing the antibody titers in patients who overcame Covid-19 and developed its own protocol for collecting plasma by manual technique using a system of multiple bags. Since plasma transfusion is a routine medical procedure, no new medical approvals are required to perform it. In fact, the same basic concept was used to treat several Ebola patients with convalescent serum during the 2014-15 epidemic.

MATERIAL AND METHODS

Specific antibody detection. Specific anti-S1S2 SARS CoV2 antibodies and anti-Nucleoprotein SARS CoV2 antibodies were quantified with home-based ELISA test. Specific SARS CoV2 S1S2 and SARS CoV2 Nucleoprotein (Sino Biological, EU) antigens were coated on polystyrene microwells (0.5 μg/ml, coat buff, 100 μl/well, overnight, 4°C). After 3 washing cycles (PBS, 0.01% Tween) microwells were blocked (PBS, 1% BSA, 1h, RT), and after further washing cycles patient samples were incubated in duplicates (1/100 diluted, 100 ul/well, 3h RT, shaking, 60 rot/min). Secondary antibodies were incubated after washing cycles (goat anti-human IgG, IgA or IgM Southern Biotech, USA, 100 ul/well, 1.5h RT, shaking, 60 rot/min). Final washing cycles were followed with substrate incubation (TMB solution, Siemens, EU, 100 ul/well, 15 min RT, dark) and after stopping (Stop solution, Siemens, EU, 50 ul/well) optical density of each sample was determined at 450nm (Synergy HT, EU spectrophotometer). Concentration of every sample was determined from the standard curves obtained with monoclonal antibodies specific for S1S2 or Nucleoprotein (Sino Biological, EU).

Patients. A total number of 950 patients were analyzed in this study, whose samples were collected in time interval from 01.05.2020. till 15.08.2020. First group were recovered patients from Covid19 hospitals (457), second group were patients from Covid19 out-clinics (311) and third group comprised family members of recovered patients (182). Serum samples frozen from volunteer healthy donors were used as a negative control (160). All control negative samples were collected in the period April – August 2019, in time period long before any signs of SARS-CoV2 pandemic.

Clinical data collection. Data were collected after fulfilling electronic questioner (www.covidmirage.com).

Convalescent plasma collection. Plasma collection was performed in patients who had undergone COVID-19, had IgG antibodies present in the circulation, and wanted to donate their plasma to treat other people. Four to six or eight weeks after infection, there should be
enough antibodies in the patient's blood to neutralize the virus and theoretically limit the infection (1). Before collecting plasma, each donor was tested for markers of transfusion transmitted infections (hepatitis B, hepatitis C, HIV 1/2 and syphilis) by Elisa tests and PCR technique, checked for the titer of Anti-SARS-CoV-2 IgG antibodies and his blood group was determined, (ABO and Rhesus factor). The procedure consists of placing a venous catheter (Cell Connect CC1, Fresenius Medical Care, Germany) into the cubital vein (most often), attaching a multiple bag system (TH, Jiaxing Tianhe Pharmaceutical Co., Ltd, China) containing 63 mL of anticoagulant-preservative CPD / SAG-M solution - composition: Citric acid - 0.299 g; Sodium citrate - 2.63 g; Monobasic Sodium Phosphate - 0.222 g and Dextrose - 2.55 g in primary bag while accompanying (satellite) bag contained 100 ml of optimal SAGM additive solution (containing: NaCl - 877 mg; Adenine - 16.9 mg; Dextrose - 900 mg and Mannitol - 525 mg), intended for resuspension of concentrated erythrocytes. After that, 450 mL of blood is taken from a voluntary donor, centrifugated (Jouan, Thermo Scientific France) at a speed of 3500 rpm for 10 minutes at a temperature of 4 ± 2° C and separated into components, ie. cell suspension and plasma, manually. During the centrifugation time, the donor is given via iv. catheter 0.9% NaCl solution to maintain cannula patency and volume recovery. In the further procedure, the cellular elements of the blood are returned to the donor after centrifugation, and after that the procedure of taking another unit of whole blood is repeated. An average of 611.47 (310-680) mL of convalescent plasma was thus collected from each donor, and the procedure took an average of 90 minutes. Plasma was frozen at -60° C within 6 hours maximum and stored in freezers at -40 ± 5 °C (Fiocchetti, Frigoriferi Scientifici, Italy) with a shelf life of three years. Whole blood (450± 45 mL) for the preparation of plasma units was taken from donors (aged 25 to 55 years), unreactive to markers of transfusion transmitted diseases (hepatitis B and C, AIDS and lues), performed by Elisa tests and PCR technique, with orderly clinical and laboratory findings.

**Statistical Analysis.** Comparison of antibody concentration between investigated groups was performed with Mann Whitney test. Values presented in Figure 1. Are given as median SD. Data analysis was performed with software package StatGraphPrism 6.

**RESULTS**

Average anti SARS-CoV2 antibody concentration in investigated groups.
As previously explained, we have quantified specific anti S1S2 Spike and anti-Nucleoprotein antibodies in Covid-19 patients with heavy or mild clinical picture. All our hospitalized patients demonstrated heavy (but not critical) clinical presentation, with average 11 days of hospital treatment. As expected, sera from hospitalized patients contained significantly more anti S1S2 Spike specific IgG (Figure 1A.) and anti-Nucleoprotein IgG antibodies (Figure 1B.) comparing to group treated out of hospital facilities. Similarly, group with severe clinical form presented significantly more anti S1S2 Spike specific IgA (Figure 1C.) and anti-Nucleoprotein IgA antibodies (Figure 1D.) comparing to less severe Covid19 group. Interestingly, group with severe Covid19 symptoms demonstrated significantly higher average concentration of S1S2 Spike specific IgM (Figure 1C.) and anti-Nucleoprotein IgM antibodies (Figure 1D.) comparing to another group.

Selection of donor’s convalescent plasma according to concentration of SARS-CoV2 specific antibodies.

Widely accepted criteria for selection of convalescent plasma donors are levels of specific anti SARS-CoV2 IgG antibodies, most frequently antibodies to envelope antigens. In our group of hospitalized patients 225 had detectable anti S1S2 Spike specific IgG antibodies, while 202 had detectable anti-Nucleoprotein IgG antibodies. According to our data, from group that presented severe Covid19, 3% of patients demonstrated extremely high concentration of anti S1S2 Spike IgG and 6% demonstrated extremely high concentration of anti-Nucleoprotein IgG antibodies, that measured more than 1000 EU/ml (Table 1.). Further 8% had high anti S1S2 Spike response and 15% had high anti-Nucleoprotein IgG levels, that measured in hundreds EU/ml. Specific anti SARS-CoV2 antibody response reflected in levels between 10-100 was detected in 26% and 28% (anti S1S2 Spike and anti-Nucleoprotein, respectively). Finally, 63% and 51% of our hospitalized Covid19 patients had modest antibody levels, ranging from 5-10 EU/ml (anti S1S2 Spike and anti-Nucleoprotein, respectively). Patients that demonstrated level of specific anti SARS-CoV2 antibodies of IgG class in hundreds or thousands were selected as convalescent plasma donors. Several patients with high concentration of specific anti SARS-CoV2 antibodies had to be excluded as potential donors according to medical indications (cardiovascular disease, hemophilia).
Time related concentration change of SARS-CoV2 specific antibodies in samples of convalescent plasma donors.

Until the end of August we have selected and collected plasma rich with specific antibodies from 12 people (12/225, 5.3% of patients cured from severe Covid19). All of these patients had at least two points of specific antibody measurements, with time interval no less than 60 days. Interestingly, concentration of anti S1S2 Spike IgG antibodies increased in 67% of our plasma donors, paralleled with increase of anti-Nucleoprotein IgG antibodies in 58% of donors (Table 2.). Concentration of anti S1S2 Spike and anti-Nucleoprotein of IgA class increased in 50% of all donors. Interestingly, while anti S1S2 Spike IgM concentration decreased in donors, concentration of anti-Nucleoprotein IgM antibodies again was increased in our plasma donors.

DISCUSSION

Convalescent plasma has a strong historical advantage and good biological value. Although this therapeutic approach is promising, it has not yet been shown to be safe in the treatment of COVID-19. Data after transfusion of ABO-compatible human convalescent plasma COVID-19 to 5,000 hospitalized adults with severe or life-threatening COVID-19, 66% of which in the intensive care unit were analyzed. The incidence of all serious adverse events, including mortality (0.3%), in the first 4 hours after transfusion was <1%. Of the 36 reported, 25 were convalescent plasma related, including mortality (n = 4), circulatory overload associated with transfusion (n = 7), acute lung injury associated with transfusion (n = 11), and severe allergic reaction after transfusion (n = 3). However, physicians estimate that only 2 of 36 reactions are definitely associated with convalescent plasma transfusion. Mortality rate after 7th day was 14.9%. Given the lethal nature of COVID-19 and the large population of critically ill patients included in these analyzes, the mortality rate does not appear to be too high. These early indicators suggest that convalescent plasma transfusion is safe in hospitalized patients with COVID-19 (13-16).

In addition to the antiviral mechanisms of neutralizing antibodies, the immunomodulatory effects of plasma components may be beneficial. Several small and large studies have shown the effects of convalescent plasma for the treatment of severe viral illness. Plasma transfusion can cause minor adverse events such as fever, nausea, allergic reactions, blood-borne pathogens transmission and some serious adverse events such as acute lung injury.
(TRALI), transfusion-associated circulatory overload (TACO) and antibody-dependent enhancement (ADE (13-19).

In June 2020, the U.S. Department of Defense began an action to collect plasma units from patients who had fully recovered from COVID-19, in order to support the development of effective treatment. The goal is to collect 10,000 units of this plasma until 10.09. 2020 (20).

There are currently no licensed vaccines or targeted therapies against the virus itself. Plasma with anti-SARS CoV-2 antibodies, obtained from recovered individuals in whom COVID-19 has been confirmed, have begun to be collected using apheresis devices and stored in blood banks in some countries to be administered to patients with COVID-19 in order to reduce the need for intensive care and a lower mortality rate. Therefore, it is necessary to point out some important issues related to convalescent plasma and its use in the treatment of patients as a form of anti-viral therapy. The protective effect can last for weeks and months. After the donor's assessment, 200-600 mL of plasma can be collected with apheresis devices (which are used in the world and in our country). The donation interval may vary between countries. Hence the necessity of testing antibody titer values. Although limited published studies are not prospective or random, until vaccination or targeted antiviral therapy is approved, plasma therapy appears to be a safe and likely effective treatment for critically ill patients with COVID-19. It can also be used for prophylactic purposes, but the safety and efficacy of this approach should be tested in randomized clinical trials and a conclusion reached (21).

Eligibility criteria for plasma donors may vary from country to country, but certainly include, above all, a safe procedure, health and antibody titers checks, and consent to the procedure. The antibody titer will vary according to the duration between the time of collection and the onset of infection. In previous studies, it has been observed that seroconversion occurs between 8 and 21 days after the onset of symptoms. In clinical trials, initially one plasma unit was given (200 mL) and repeated after 12 hours. The duration of antibody efficacy is not known, but it is estimated that it will last for weeks to several months (21-27). On August 23, 2020, the Food and Drug Administration (FDA) issued an approval for the emergency use of convalescent plasma COVID-19, for the treatment of hospitalized patients with COVID-19. There are insufficient data to recommend for or against the use of convalescent plasma for the treatment of COVID-19. Available data suggest that serious adverse reactions after administration of COVID-19 convalescent
plasma are rare and consistent with the risks associated with plasma infusions in other indications. The long-term risks of treatment with convalescent plasma COVID-19 and whether its use reduces the immune response to SARS-CoV-2, making patients more susceptible to re-infection, have not been assessed. Convalescent plasma should not be considered the standard of care for the treatment of patients with COVID-19. Prospective, well-controlled, adequately initiated randomized trials are needed to determine whether convalescent plasma is effective and safe for the treatment of COVID-19. All this confirms the fact that it is necessary to collect and store certain quantities of convalescent plasma in order to provide reserves, which encouraged us to provide plasma reserves for the treatment of COVID-19 patients with modest funds and without additional costs.

CONCLUSION

Originally developed ELISA tests enable novel protocol for selection of convalescent blood plasma donor. According to our data, it is necessary to recruit and test a large number of patients recovered from severe Covid19 in order to have sufficient number of appropriate convalescent plasma donors.

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TABLES AND FIGURES

Table 1. Identification of potential convalescent plasma donors according to concentration of specific anti SARS-CoV2 antibodies.

| Ab Conc    | anti S1S2 Spike IgG | anti- Nucleoprotein IgG |
|------------|---------------------|-------------------------|
|            | patients            | patients                |
| > 1000 EU/ml | 5                  | 10                      |
|            | 3 %                 | 6 %                     |
| > 100 EU/ml | 19                 | 31                      |
|            | 8 %                 | 15 %                    |
| > 10 EU/ml  | 59                 | 57                      |
|            | 26 %                | 28 %                    |
| 5-10 EU/ml | 142                | 104                     |
|            | 63 %                | 51 %                    |

Table 2. Time related concentration change of SARS-CoV2 specific antibodies in samples of convalescent plasma donors.

| donor | anti S1S2 Spike IgG | anti- Nucleoprotein IgG |
|-------|---------------------|-------------------------|
|       | IgG | IgA | IgM | IgG | IgA | IgM |
| 617   | ▲   | ▲   | ▼   | ▼   | ▼   |
| 666   | ▲   | ▲   | ▼   | ▼   | ▼   |
| 778   | ▲   | ▲   | ▲   | ▲   | ▲   |
| 664   | ▲   | ▲   | ▼   | ▼   | ▼   |
| 12    | ▲   | ▼   | ▲   | ▼   | ▼   |
| 956   | ▼   | ▼   | ▼   | ▼   | ▼   |
| 256   | ▼   | ▼   | ▼   | ▼   | ▼   |
| 950   | ▼   | ▼   | ▼   | ▼   | ▼   |
| 1030  | ▼   | ▼   | ▼   | ▼   | ▼   |
| 367   | ▼   | ▼   | ▼   | ▼   | ▼   |
| 119   | ▼   | ▼   | ▼   | ▼   | ▼   |
| 1065  | ▼   | ▼   | ▼   | ▼   | ▼   |
| 617   | ▼   | ▼   | ▼   | ▼   | ▼   |
| increase | 67 % | 50 % | 42 % | 58 % | 50 % | 58 % |
Figure 1. Average concentration of SARS-CoV2 specific antibodies in investigated patients.
