Original article

A non-randomized multicentre trial of human immune plasma for treatment of hantavirus cardiopulmonary syndrome caused by Andes virus

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Background: In Chile, Andes virus (ANDV) is the sole aetiological agent of hantavirus cardiopulmonary syndrome (HCPS) with mean annual incidence of 55 cases, 32% case fatality rate (CFR) and no specific treatment. Neutralizing antibody (NAb) titres at hospital admission correlate inversely with HCPS severity. We designed an open trial to explore safety and efficacy and evaluate pharmacokinetics of immune plasma as a treatment strategy for this disease.

Methods: We performed plasmapheresis on donors at least 6 months after HCPS and measured NAb titres through a focus-reduction neutralization test. Subjects admitted to 10 study sites with suspected/confirmed HCPS were eligible for treatment with immune plasma by intravenous infusion at an ANDV NAb dose of 5,000 U/kg. HCPS was confirmed through immunoglobulin M serology or reverse transcriptase-PCR. The main outcome was mortality within 30 days.

Results: From 2008–2012, we enrolled and treated 32 cases and confirmed HCPS in 29. CFR of hantavirus plasma-treated cases was 4/29 (14%); CFR of non-treated cases in the same period in Chile was 63/199 (32%; *P*=0.049, OR=0.35, CI=0.12, 0.99); CFR of non-treated cases at the same study sites between 2005–2012 was 18/66 (27%; (*P*=0.15, OR=0.43, CI=0.14, 1.34) and CFR in a previous methylprednisolone treatment study was 20/60 (33%; *P*=0.052, OR=0.32, CI=0.10, 1.00). We detected no serious adverse events associated to plasma infusion. Plasma NAb titres reached in recipients were variable and viral load remained stable.

Conclusions: Human ANDV immune plasma infusion appears safe for HCPS. We observed a decrease in CFR in treated cases with borderline significance that will require further studies for confirmation.

Introduction

Hantavirus cardiopulmonary syndrome (HCPS) is a zoonosis occurring throughout the Americas and affecting mainly rural inhabitants [1–4]. The infection produces a capillary leak syndrome with lungs as the main target organ, whereas death usually results from cardiogenic shock [5]. Andes virus (ANDV) is the only identified hantavirus in Chile with a mean annual incidence rate of 55 cases and a case fatality rate (CFR) of 32% [6,7]. Person-to-person transmission of ANDV, which has been documented in both Chile and Argentina, is unique among hantaviruses [8–10].

There are no drugs with proven efficacy for HCPS. Treatment is based on critical care support, including extracorporeal membrane oxygenation (ECMO) [11,12]. Ribavirin has benefit in haemorrhagic fever with renal syndrome but failed to show benefit in

HCPS [13,14]. A DNA vaccine for ANDV is in preclinical studies [15,16].

While the role of cellular immune response in control of viral replication and in disease pathogenesis is controversial [17–19], humoral immune response, directed to envelope glycoproteins Gn and Gc is enough for protection from infection and administration of immune plasma before day 5 after infection prevented death in 94% of the animals in the ANDV Syrian hamster model [20,21]. In humans with HCPS caused by Sin Nombre virus (SNV), neutralizing antibody (NAb) titres at hospital admission correlate inversely with disease severity [22].

Considering that NAb titres for SNV and ANDV remain high for years after disease, that viral RNA is not detected by reverse transcriptase (RT)-PCR on plasma obtained months after HCPS [23–25], the Syrian hamster results and the correlation of NAb titres with prognosis in HCPS, we hypothesized that immune plasma obtained from ANDV survivors could be administered safely to HCPS cases and may be a therapeutic option. This approach used in the arenavirus disease Argentine haemorrhagic fever (AHF) decreased lethality from 20–25% to 1% if infused within 7 days of disease onset. To explore safety and efficacy of ANDV immune plasma and evaluate ANDV NAb pharmacokinetics, we developed an open, compassionate use protocol for treatment of HCPS using human immune plasma from ANDV disease survivors.

Methods

Ethics statement

All human subject participation was in compliance with the Declaration of Helsinki and Chilean regulations. The protocols for immune plasma donation and treatment with immune plasma were approved by the Ethics Committee of Clínica Alemana-Universidad del Desarrollo School of Medicine and local ethics committees as required by clinical sites. Written informed consent was obtained from plasma donors and from participant subjects or their next of kin in case of incapacity because of HCPS. In minors, consent was given by one of their parents, with assent from the minor when possible.

Development of immune plasma banks

Adults with previous ANDV infection were plasmapheresed at the Clínica Alemana blood bank. Inclusion criteria for plasma donation were age ≥18 years, positive immunoglobulin (Ig)G serology, ≥6 months since recovery from HCPS or having received any blood product, ≥8 months since ECMO and compliance with national criteria for donor screening. Exclusion criteria included any contraindication for plasmapheresis. A total of 700–800 ml plasma were obtained at each donation. Plasma was classified by blood group, screened for HIV, HBV, HCV, human T-lymphotropic virus (HTLV) I-II, Chagas and syphilis and stored in bags of approximately 250 ml at -80°C at the blood bank. ANDV NAb titres for plasma samples were measured through a plaque reduction assay [22]. Bags of plasma with neutralizing titres ≥1:400 and different blood groups were stored at blood banks in Santiago, Valdivia and Puerto Montt.

Treatment of cases of HCPS with immune plasma

Research teams with intensive care unit physicians and nurses were established at 10 public or private hospitals in three cities in Chile. When a hantavirus suspected or confirmed case presented in a site, a member of the investigator team evaluated the patient for inclusion criteria and obtained written informed consent. The inclusion criteria were, for confirmed hantavirus diagnosis: positive IgM or RT-PCR for hantavirus in blood plus acute febrile illness of <12 days; for presumptive hantavirus diagnosis all of the following: <12 days of disease characterized by fever; headache or myalgia or nausea, vomiting, diarrhoea or abdominal pain; platelet count $<150\times10^3$/ml; immunoblasts in peripheral blood if available; hypoxia. Exclusion criteria were, in case of presumptive hantavirus infection, other probable or confirmed diagnosis; history of severe adverse reaction to human plasma administration; history of IgA deficiency.

Immune plasma infusion

We administered blood group compatible plasma at a dose of 5,000 units of NAb per kg of body weight; in patients subjected to ECMO we administered a second 5,000 units/kg dose. A unit was defined as the reciprocal of the end point NAb titre multiplied by the volume in ml. Plasma was infused by the site staff at 1–2 ml/min for 10 min followed for 3–6 ml/min according to tolerance.

Subject evaluation and follow-up

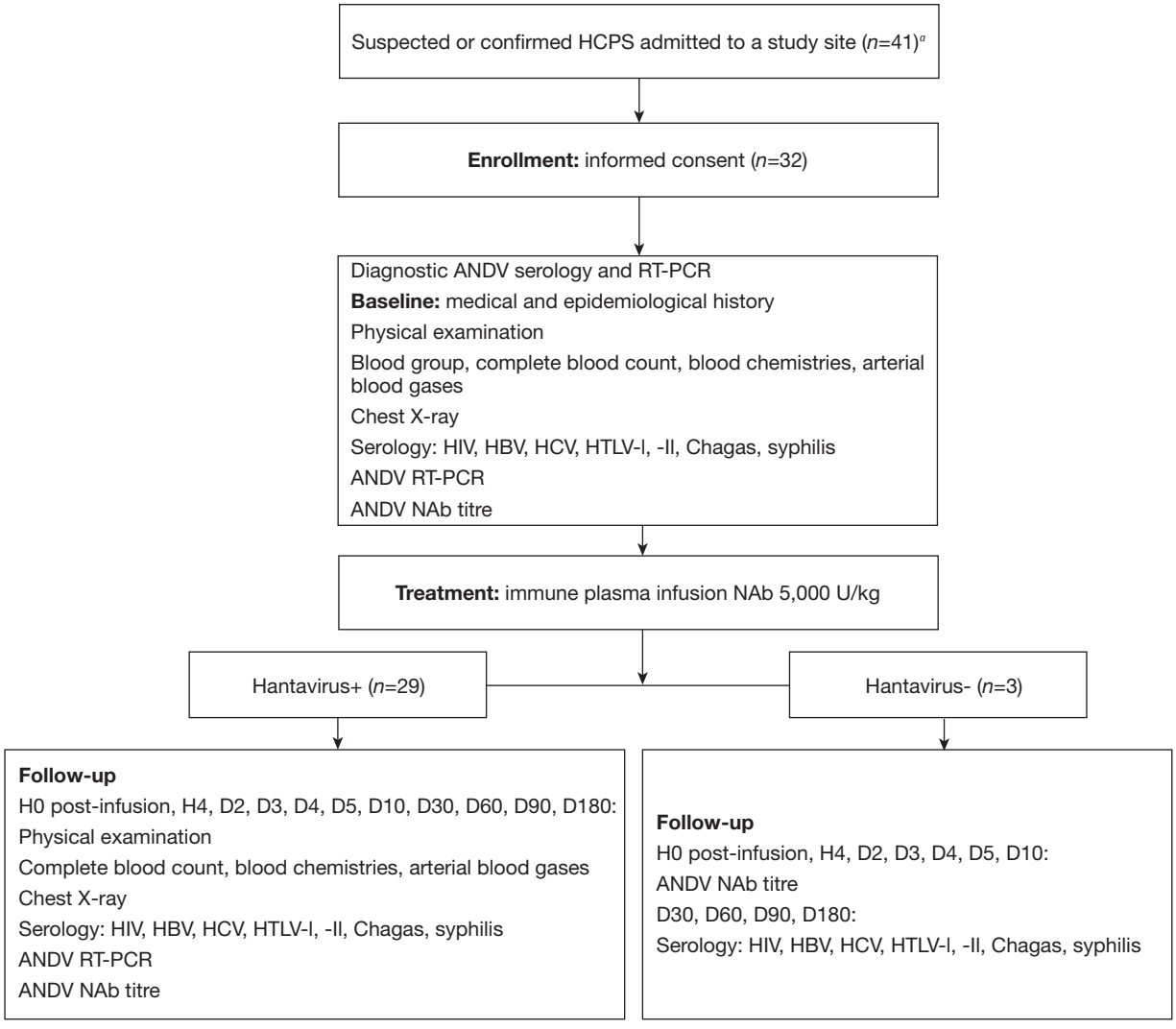
At enrolment we carried out a clinical history, physical examination, chest radiograph and obtained samples for hantavirus diagnostic confirmation, blood group, complete blood cell count (CBC), blood gases, blood chemistries, liver function tests, serum lactate and serology for blood-borne infections (HIV, HBV, HCV, HTLV I-II, Chagas and syphilis). ANDV quantitative RT-PCR (qRT-PCR) and NAb titres were measured at 0, 4 and 24 h post-infusion and on days 3, 4, 5, 10, 30, 90 and 180. Follow-up also included CBC, blood gases, blood chemistries, liver function tests, serum lactate, coagulation tests and chest radiograph at 4 and 24 h, days 3, 4, 5, 10 and days 30, 90 and 180

if still abnormal. Serological testing for blood-borne infections was repeated on days 30 and 90 and for HCV on day 180. Serious adverse events (SAE) were classified according to the CTCAE table of National Cancer Institute, version 3.0, March 2003 [26] (Figure 1).

Hantavirus serology and quantitative RT-PCR

Serum samples were tested for IgG and IgM antibodies to recombinant ANDV antigens by ELISA [27]. qRT-PCR was performed in peripheral white blood cells (WBC) obtained by centrifugation of 4 ml of whole blood with EDTA. After centrifugation, buffy coat was removed with a small volume of plasma (approximately 200 µl) and total RNA was extracted using a High Pure Viral Nucleic Acid kit (Roche Diagnostics, Mannheim, Germany). RNA (5 µl) was used as a template for cDNA synthesis with the primer S 5′-GCAGCTGTGTCTA-CATTGGAGAC-3′. Taqman PCR assay was carried out using cDNA, primers and probes as described, and adapted for the LightCycler v3.5 (Roche Diagnostics) [28]. All PCR reactions were performed using the

Figure 1. Study flowchart



[a]Reasons for non-enrolment in the study: late suspicion and diagnosis (*n*=3), investigation team not available (*n*=2), death shortly after hospital admittance (*n*=2), mild disease (*n*=1) and unknown to the investigation team (*n*=1). ANDV, Andes virus; D, day; H, hour; HCPS, hantavirus cardiopulmonary syndrome; HTLV, human T-lymphotropic virus; NAb, neutralizing antibody; RT-PCR, reverse transcriptase PCR; +, positive; -, negative.

Light Cycler TaqMan Master® kit (Roche Diagnostic). Amplification products were determined by continuous fluorescence monitoring. For quantification, serial dilutions of a plasmid containing the PCR target region were used as a standard curve. The RNA amount was normalized to WBC in the sample. The WBC were quantified by amplification of endogenous housekeeping β-globin gene using SYBR green real-time PCR as described [29]. The RNA amount was expressed as copies of ANDV S-segment per 10^7 WBC, calculated as the ratio of ANDV S-segment copy number/[β-globin DNA copy number/2]. In a subgroup of eight cases qRT-PCR was also performed in plasma using the same method and expressed as ANDV S-segment copy number per ml of plasma.

NAb titres to ANDV

NAb titres were measured as described [22]. Serial twofold dilutions from 1:100 to 1:6,400 of heat-inactivated patient plasma were mixed with equal volume of approximately 50–100 focus-forming units per ml of ANDV (Chilean strain of human origin, isolate CHI-7913) and incubated at 37°C for 1 h. The mixture was then used to infect a confluent monolayer of Vero E6 cells (ATCC CRL 1586) in duplicate wells of a 48-well dish, with a 1.2% methylcellulose overlay in the medium to confine the virus to the foci. After incubation for 1 week, viral foci were detected with polyclonal rabbit anti-N antibody followed by peroxidase-conjugated goat anti-rabbit immunoglobulin G. NAb titres were defined as the reciprocal of the highest serum dilution that resulted in an 80% reduction in the number of foci compared to virus controls in duplicate assays.

Statistical analysis

The study was designed to compare CFR in the treatment group versus: untreated cases diagnosed during the enrolment period in the whole country; untreated patients admitted to the same sites from 2005 to 2012, in order to control for site biases; and well characterized patients enrolled in a previous trial of methylprednisolone (MP) for HCPS, from our group, to control for severity at entry [30]. We estimated a sample size of 35 cases to detect a 75% reduction compared to national CFR with 5% alpha error and 80% power. Reporting of hantavirus disease in Chile is mandatory and site and national mortality rates were available through the Ministry of Health.

We performed descriptive analysis using frequency distribution and percentages for categorical variables and central tendency statistics for continuous variables. For comparisons of continuous variables we used mean and SD and t-test for significance or, for variables not normally distributed, median, IQR and Mann–Whitney U test. Viral RNA kinetic curves were compared through a general linear model. We used a Kaplan–Meier model and log-rank test for survival analysis according to severity at entry. Analyses were carried out with SPSS version 19.0 (SPSS Inc., Chicago, IL, USA) and EPIDAT 3.1 (PAHO/WHO).

Results

Between October 2004 and January 2012 we performed plasmapheresis once or twice on 32 donors resulting in a total of 44 donations with a mean volume of 785 ml per donation. ANDV NAb titres ranged from 1:200 to >1:6,400. Screening for blood-borne pathogens was negative, and plasma from all but one donor was considered safe for use in humans. Between 1 January 2008 and 12 March 2012 we enrolled 32 patients at 6 of the 10 sites (3 public, 3 private) and confirmed acute hantavirus infection in 29 (Additional file 1). No cases occurred in the other sites. During the same period in Chile, another 199 cases of hantavirus disease were reported to the Ministry of Health. Nine of these case-patients were admitted to a study site but not enrolled. Reasons for non-enrolment were: suspicion and diagnosis during recovery phase (n=3), investigation team not available (n=2), death shortly after hospital admittance (n=2), mild disease (n=1) and unknown to the investigation team (n=1). These cases were included in the non-treated group for the analysis (Figure 1).

A total of 15 cases were male and 14 female, with a median age of 34 (range 5–73); 76% were Hispanic, 14% Amerindians and 10% other; 21 had at least one medical visit relating to their present illness before the visit leading to hospitalization and 17 (59%) cases were transferred to the study site from a smaller hospital. The median number of days between first symptoms and hospital admission was 6 (range 1–12). All received the total dose of NAbs as per protocol. The median time between admission to the site hospital and plasma infusion was 14.7 h (range 3.2–103.8) and median infusion time was 1.3 h (range 0.3–9.6). Four patients received a second 5,000 U dose because of ECMO. At entry, 21 had mild–moderate disease (sequential organ failure assessment [SOFA]≤8) and eight had severe disease (SOFA>8). Four cases were placed on ECMO, two of whom died. All three enrolees without hantavirus infection (one endocarditis, one leptospirosis and one undetermined) survived.

CFR of treated versus untreated patients during study period nationwide

A total of 4 of the 29 enrolled subjects with confirmed hantavirus died (14%). In contrast, there were 63 deaths among 199 (32%) confirmed HCPS cases reported to the Ministry of Health during the same period who did not receive immune plasma (P=0.049; OR=0.35, CI=0.12, 0.99; Table 1).

CFR of treated versus untreated patients at same study centres from 1 January 2005 to 12 March 2012

To determine if there was an effect of the study centres on CFR, we compared the CFR of enrolled versus non-enrolled HCPS cases hospitalized in the study sites between 1 January 2005 and 12 March 2012. There were 18 deaths in 66 (27%) untreated cases compared with 4/29 treated cases (*P*=0.15; OR=0.43; CI=0.14, 1.34; Table 1).

CFR of treated versus untreated patients from the MP study

Between 2003–2010 we conducted a double-blind, placebo-controlled clinical trial to evaluate safety and efficacy of intravenous MP for HCPS. We enrolled 60 confirmed cases, of whom 30 received MP and 30 received placebo. MP treatment was safe but did not provide significant clinical benefit to the patients [30]. We compared the CFR of the present study versus cases enrolled in the MP study to reduce bias from inability to enrol patients who die before transfer to a study centre or before evaluation for study entry. Clinical data was systematically collected in both studies. Demographics and clinical parameters at entry of cases in both studies were similar (Table 2). The CFR in the MP study was higher 20/60 (33%) than that of the present study with borderline significance (*P*=0.052; OR=0.32; CI=0.10, 1.00).

In the MP study, disease severity at study entry based on the SOFA score was the best predictor of survival. To further evaluate the effect of disease severity at entry on survival in the present study, we compared the survival in the MP study with survival in the current study after stratification by SOFA score at entry (Figure 2 and Table 3). Using this comparison, while mortality was significantly higher in both studies in subjects who had severe disease at entry (SOFA>8), there was no significant difference in mortality between the two studies after stratification for disease severity at entry.

Safety of immune plasma

We detected 11 SAEs including the 4 deaths, none of them associated with immune plasma. Three deaths were attributed directly to HCPS. The fourth died on day 20 from complications of ECMO (extensive necrosis of lower limb and secondary bacterial sepsis). During follow-up, no immune plasma recipient had clinical or laboratory evidence of acquisition of any blood-borne pathogen.

Neutralizing antibody kinetics

In the three patients who tested negative for hantavirus, infusion of immune plasma resulted in NAb titres

Table 1. CFR of treated versus untreated confirmed hantavirus cases

	Deaths, *n*	Total, *n*	CFR, %	*P*-value	OR (CI)
Cases treated with immune plasma					
Study site[a] 2008–2012	4	29	14	–	–
Cases not treated with immune plasma					
Whole country 2008–2012	63	199	32	0.049	0.35 (0.12, 0.99)
Same sites[a] 2005–2012	18	66	27	0.152	0.43 (0.14, 1.34)
Methylprednisolone study	20	60	33	0.052	0.32 (0.10, 1.00)

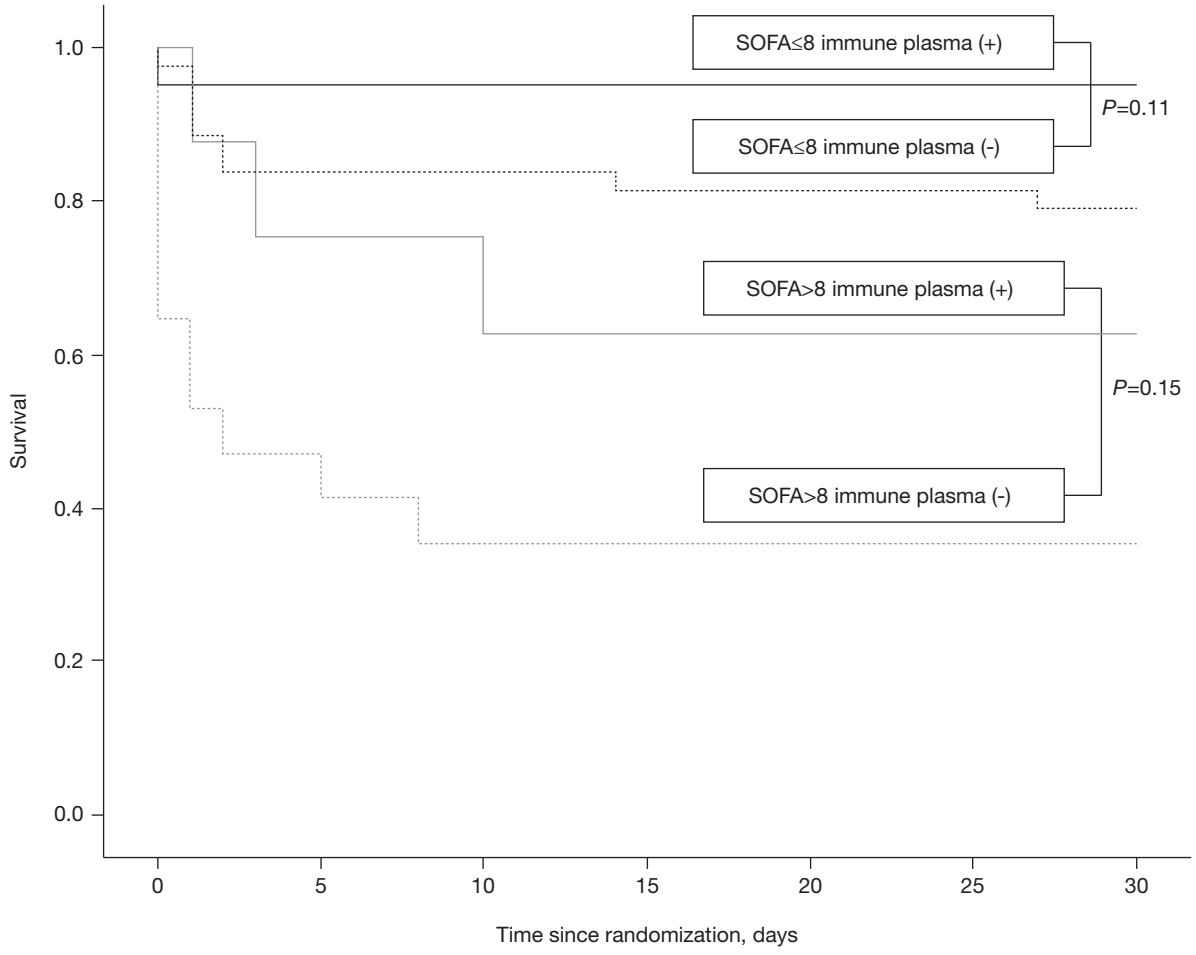
[a]Study sites that enrolled cases were Hospital de Valdivia, Clínica Alemana de Santiago, Hospital de Puerto Montt, Clínica Las Condes, Clínica Santa Maria and Hospital Padre Hurtado. Four sites that did not enrol cases where not included. CFR, case fatality rate; OR, odds ratio.

Table 2. Comparison between cases enrolled in the methylprednisolone study and immune plasma study at entry

	Methylprednisolone study (*n*=60)	Immune plasma study (*n*=29)	*P*-value
Median age, years (IQR)	40.4 (30.5–50.4)	34.3 (22.3–44.1)	0.16
Male sex, *n* (%)	42 (70)	15 (52)	0.11
Median time from symptom initiation and enrolment, days (IQR)	6 (4–8)	6 (4.5–8)	0.65
Median PaO_2/FiO_2 ratio (IQR)	171.9 (113.7–253.1)	173.5 (116.8–248.0)	0.86
Intubation, *n* (%)	14 (23)	8 (28)	0.79
Shock[a], *n* (%)	24 (40)	11 (38)	1.00
Median platelet count $\times 10^3/\mu l$ (IQR)	56.7 (33.0–84)	57.0 (40.0–91.5)	0.65
Median SOFA score (IQR)	6.5 (5.0–8.9)	5.0 (3.6–8.7)	0.08

[a]Shock is defined as median blood pressure <70 mmHg or use of vasoactive drugs. FiO_2, fraction of inspired oxygen; PaO_2, partial pressure of arterial oxygen; SOFA, sequential organ failure assessment.

Figure 2. Kaplan–Meier survival analysis according to administration of immune plasma and severity at entry



Cases who did not receive immune plasma are all cases included in the methylprednisolone trial. SOFA, sequential organ failure assessment.

Table 3. Number at risk for survival analysis

Day	0	3	6	9	12	15	18	21	24	27	30
SOFA≤8 immune plasma (+)	21	20	20	20	20	20	20	20	20	20	20
SOFA≤8 immune plasma (-)	43	36	36	36	36	35	35	35	35	35	34
SOFA>8 immune plasma (+)	8	7	6	6	5	5	5	5	5	5	5
SOFA>8 immune plasma (-)	17	8	7	6	6	6	6	6	6	6	6

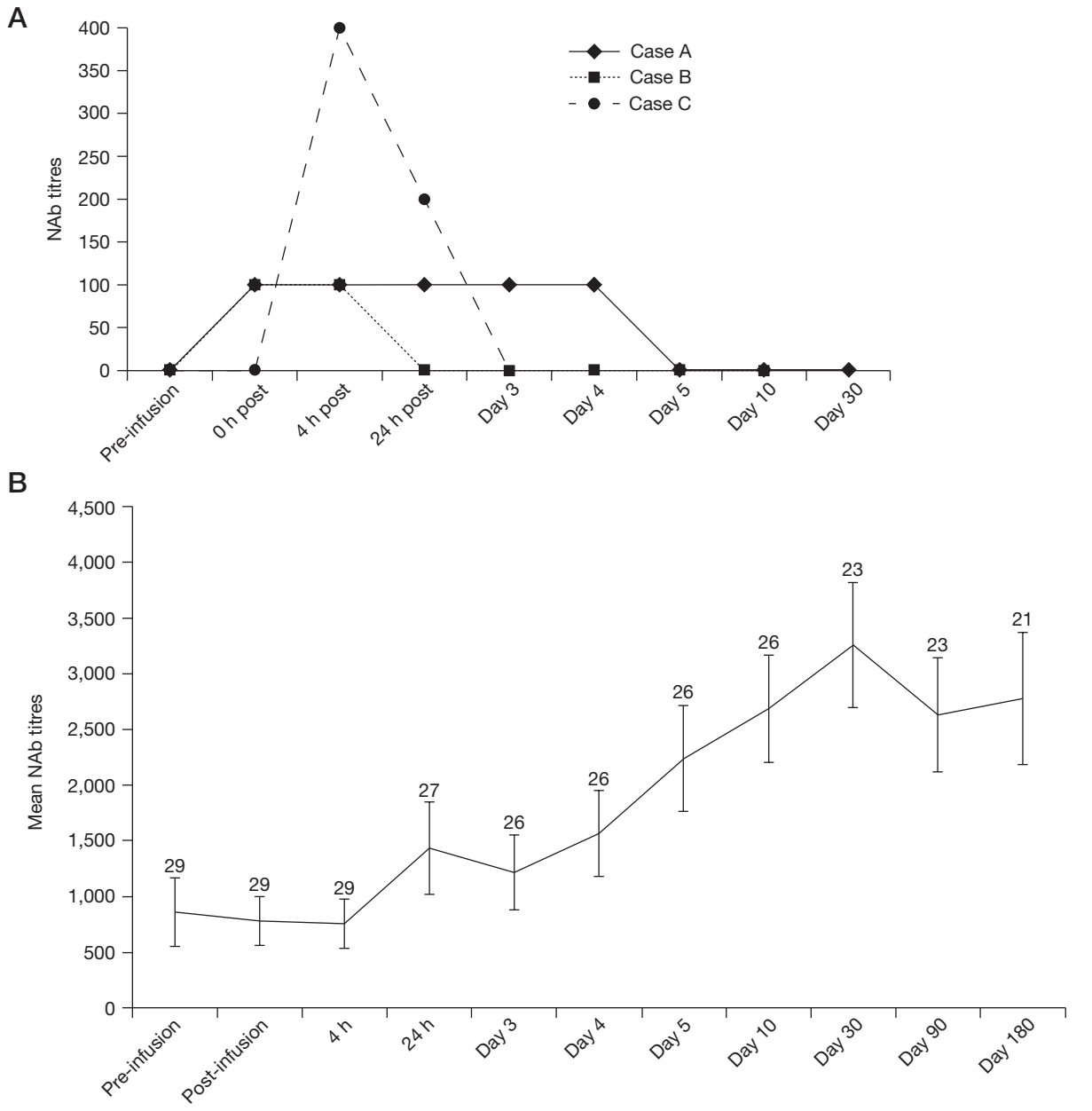
Values are number at risk. SOFA, sequential organ failure assessment.

of 1:100 to 1:400 for periods ranging from 4 h to 4 days (Figure 3A). In 26/29 confirmed cases, NAb were detected at admission in ranges from 1:100 to >1:6,400. There was no significant difference in median entry NAb titres between patients who died and survivors. In 16/29 NAb titres increased in samples taken immediately or 4 h after plasma infusion, in 8 they remained unchanged and in 5 they decreased. The mean NAb titre kinetics are shown in Figure 3B. Progressive increases in NAb titres after day 3 result from patients' own humoral immune responses.

ANDV qRT-PCR in WBC and plasma

We performed ANDV qRT-PCR in WBC from all cases and in plasma in a subgroup of eight cases. There was no correlation between viral load at admission and disease severity (including death, shock or worst SOFA score). ANDV RNA levels in WBC remained stable after

Figure 3. Plasma kinetics of neutralizing antibody titres



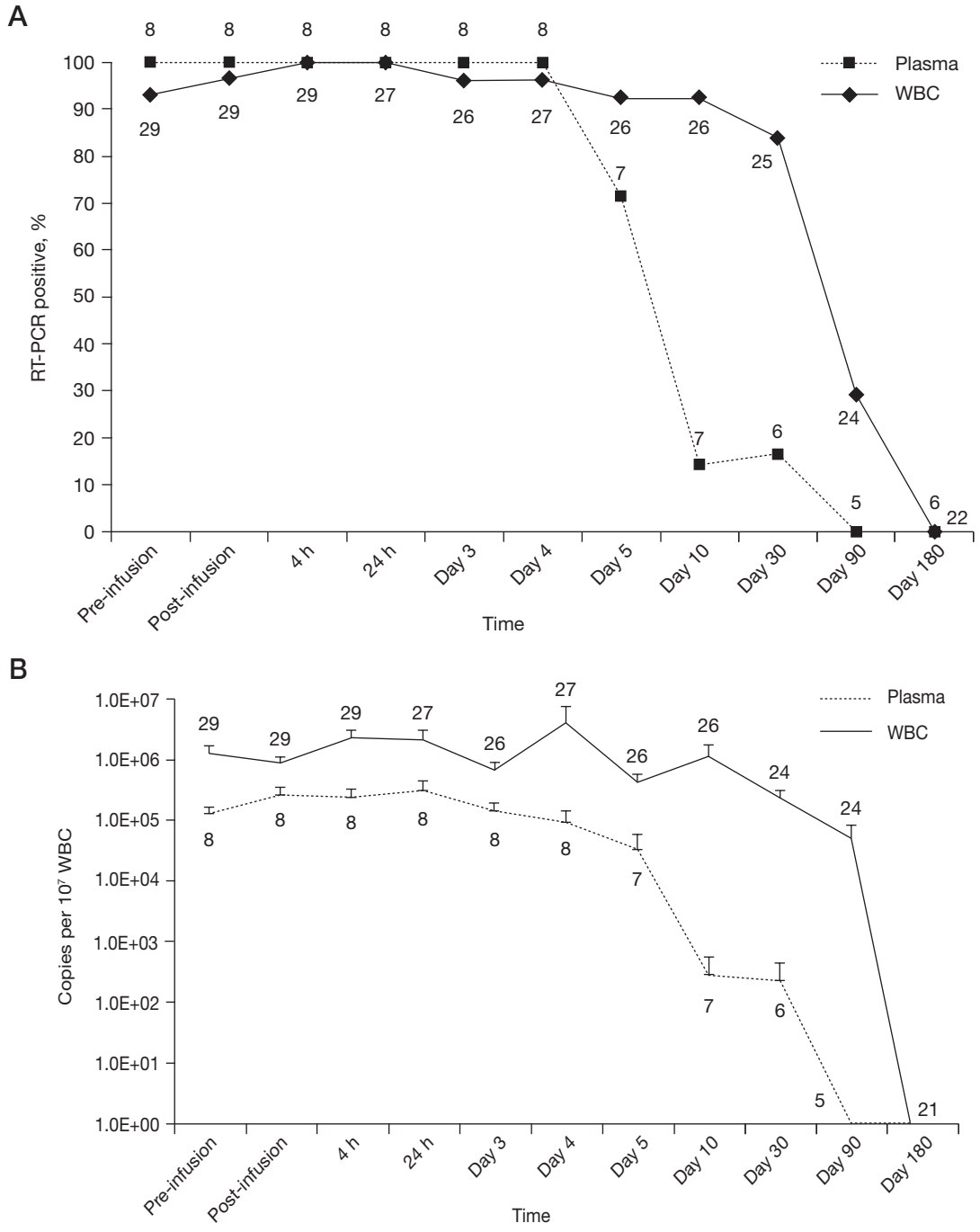
(A) Plasma kinetics of neutralizing antibody (NAb) titres in three hantavirus-negative subjects treated with immune plasma in NAb dose of 5,000 U/kg. **(B)** Plasma kinetics of NAb titres in 29 hantavirus-positive cases treated with immune plasma in NAb dose of 5,000 U/kg. Numbers above each point represent number of cases. Bars = standard error.

immune plasma administration and began to decline after day 30; in contrast, viral RNA levels in plasma declined rapidly after day 5 of clinical course (Figure 4). Viral RNA kinetics were compared with published data from our study of MP versus placebo in HCPS [30]. We did not find significant differences in the viral RNA kinetics during the first 30 days of clinical course. However in the samples obtained at day 90 after admission, 7/24 (29%) cases of immune plasma recipients versus 12/14 (86%) of the MP study remained with detectable viral load in WBC (P=0.002).

Discussion

In this study, administration of human immune plasma with ANDV NAb appeared to be safe when administered in confirmed or suspected HCPS. We observed a reduction in CFR of immune plasma treated cases to

Figure 4. Viral detection and viral load



(A) Percentage of cases with positive reverse transcriptase (RT)-PCR for Andes virus (ANDV) in white blood cells (WBC) and plasma before immune plasma infusion and during follow-up. **(B)** Quantitative RT-PCR for ANDV in WBC and plasma. Numbers above or below each point represent number of cases. Bars = standard errror.

14%, which has borderline statistical significance when compared to the CFR of 32% in the rest of the country during the study period. A tendency to lower CFR was also observed when compared to the historical lethality of untreated cases in the same study sites and to comparable cases enrolled in the MP protocol [30].

Although NAb reached detectable levels in all three patients without ANDV infection, the titres were

relatively low and of short duration. Since surviving patients develop high NAb titres within a few days of hospital admission, the longer persistence of passively administered NAb may not be a critical issue in this strategy. However, dose-ranging studies should be considered for evaluation of future NAb products to ensure that passive administration leads to consistently high levels of plasma NAb. In hantavirus-positive cases, almost all of whom had detectable NAb at entry, we detected an increase in titres in the majority immediately after plasma infusion, but only from day 3 on we observed a progressive and sustained rise in titres, probably reflecting patients' own NAb production. These results, too, suggest that higher NAb doses may be required to achieve higher titres during the first 48 h when most deaths occur. Infusion of NAb did not reduce WBC or plasma ANDV RNA levels. Because of the difficulty in culturing hantaviruses from human samples, it was not possible to determine whether infectivity was decreased by administering NAb. ANDV RNA remained detectable in WBC until day 90 in a significant proportion of cases, confirming previous observations that virus RNA remains detectable in WBC long after recovery from HCPS [30,31].

There are important limitations to this study. The best predictor of survival from HCPS is disease severity at entry, and it is exceedingly difficult to control for this and other factors influencing survival in the absence of an adequately powered, randomized, controlled trial. Comparison with national CFR suggests benefit from immune plasma treatment and the same tendency is observed when compared with rates in the current study centres and in the MP study. When stratified for disease severity at entry there is no significant difference with the MP study.

Another limitation is standardization of the study product. Plasma collection from survivors of ANDV infection and measurement of NAb titres from each plasma unit was the only practical option available for the current study. A standardized product with high NAb titre that can be produced in large quantity should ideally be used for future studies.

While treatment with immune plasma in AHF reduced mortality from 20% to 1%, timely administration of immune plasma was possible because of a prolonged prodromal phase in AHF. Early NAb administration was an important factor in the ANDV Syrian hamster model: passive administration of NAb significantly reduced mortality in hamsters when administered up to 5 days after ANDV challenge, however, protection decreased markedly near disease onset. HCPS has a relatively short febrile prodrome that is difficult to differentiate on clinical grounds from other febrile illnesses. Patients are usually hospitalized only a few hours before or after the onset of the cardiopulmonary phase, and most deaths occur within 24 h of hospital admission [30]. As such, there is a very narrow window for intervention.

Despite the open study design and the inability to determine efficacy, the results of this study are promising and should stimulate further research. Because it is not clear how closely the hamster model mimics disease in humans, the recent development of a non-human primate model of New World hantavirus infection in Rhesus macaques [32], may provide a better opportunity to evaluate the effectiveness of passive NAb administration after disease onset in an animal model.

Acknowledgements

In addition to the authors, members of The Hantavirus Study Group in Chile who contributed to patient enrolment, follow up, sample collection and analysis are as follows: Marlis Täger, Carola Osorio and Veronica Yobanolo (Hospital de Valdivia); Andrea Carriel, Paola Lanino, Alejandra Demian, Catherine Bosnich, (Hospital de Puerto Montt); Sergio Valdes (Clinica Las Condes, Santiago); Guillermo Nuñez, Cecilia Cornejo (Clínica Santa Maria, Santiago); Jan Wilhelm, Alfredo Umaña, (Clínica Alemana de Santiago, Hospital Padre Hurtado); Fernando Nagano, Maria Teresa Gonzalez, (Clínica Alemana de Santiago); Susana Rios, Edith Belmar (Facultad de Medicina Clínica Alemana Universidad del Desarrollo); and Marcela Ferres (Pontificia Universidad Catolica de Chile).

This work was supported by the Chilean government through Fondo Nacional de Investigación en Salud FONIS (SAO7120045). Immune plasma collection was also supported partially by the National Institute of Allergy and Infectious Diseases at the National Institutes of Health (5U19AI045452).

Disclosure statement

The authors declare no competing interests.

Additional file

Additional file 1: A figure of the number of enrolled cases by site can be found at http://www.intmedpress.com/uploads/documents/3230_Vial_Addfile.pdf

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Accepted 12 September 2014; published online 15 October 2014