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In vitro and in vivo validation of stored swine erythrocyte viability to establish an experimental model of homologous red blood cell transfusion: a pilot study

Validação in vitro e in vivo da viabilidade de eritrócitos suínos estocados para estabelecer um modelo experimental de transfusão homóloga de hemácias: estudo piloto

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ABSTRACT

Objective: To develop experimental models of erythrocyte transfusion, the first step is to ensure the viability of the red blood cells transfused. In this pilot study, we assessed the viability of transfused red blood cells with validation in vitro and in vivo of homologous swine erythrocytes stored for 14 days.

Methods: Blood collected from one Agroceres® swine was stored in two red blood cell units. In vivo validation was performed by labeling the red blood cells with $Na_2^{51}CrO_4$ and recovering the viable erythrocytes after 24 hours of infusion in one autologous and four homologous animals. In vitro validation was performed at baseline and after 14 days in sixteen red blood cell units by measuring hemoglobin, hematocrit, hemolysis index and free hemoglobin. A post-mortem splenectomy was performed to evaluate the splenic sequestration of erythrocytes, and the radioactivity of the supernatant samples was counted to evaluate intravascular hemolysis.

Results: After 14 days of storage, the red blood cell units had lower volumes and equivalent total concentrations of hemoglobin and hematocrit compared to human standards. The free hemoglobin concentration increased from 31.0±9.3 to 112.4±31.4mg/dL (p<0.001), and the hemolysis index increased from 0.1±0.1 to 0.5±0.1% (p<0.001). However, these tests were within the acceptable range for human standards. The percentage of radioactivity in supernatant samples was similar at baseline and after 24 hours, thus excluding significant hemolysis. No evidence of splenic sequestration of radioactive erythrocytes was found.

Conclusion: Swine red blood cells stored for 14 days are viable and can be used in experimental studies of transfusion. These validation experiments are important to aid investigators in establishing experimental models of transfusion.

Keywords: Transfusion medicine; Erythrocytes; In vitro; Swine; Validation studies

INTRODUCTION

Red blood cell (RBC) transfusion is an established treatment for multiple life-threatening conditions. However, several concerns have been raised regarding the association of transfusion with harmful effects and increased mortality,[1-4] even though the literature is discordant on this topic.[5,6] The mechanisms and clinical effects of transfusion are mainly described in observational studies, which carry an unavoidable potential for bias and residual confounders, including disease severity, presence of frequent comorbidities (such as sepsis and trauma), number of units transfused and length of storage. Despite the use of statistical techniques that may adjust for the confounders, it is very difficult

to establish the independent role of transfusion in adverse outcomes in these settings.[7]

Experimental models are increasingly being used to evaluate the mechanistic effects of interventions commonly performed in critical care.[8] Hence, the swine model may be an important tool to study the hemodynamic and respiratory effects of transfusion in critically ill scenarios, because these animals' hemodynamic and respiratory systems are physiologically similar to those of humans.[7] Therefore, a controlled animal model of RBC transfusion in healthy swine could be an interesting approach to carry out mechanistic studies and evaluate the isolated effects of RBC transfusion, without the confounding variables that are present in clinical trials.

To adequately develop an experimental model of transfusion, the first step is to ensure that the erythrocytes transfused are viable. Thus, we carried out this pilot study to assess the viability of swine RBC that were stored for 14 days using standard human procedures and then used for transfusion, with validation in vitro and in vivo.

METHODS

This study was carried out at the *Instituto de Ensino e Pesquisa* of the *Hospital Sírio-Libanês* and was approved by the Institutional Animal Research Ethics Committee. It was conducted according to the National Institute of Health guidelines for the use of experimental animals.

In vivo blood validation

To assess the feasibility of swine RBC collection and storage under usual blood bank conditions and to validate RBC survival in vivo, we conducted a pilot experiment in which a male Agroceres® pig (50kg) was maintained under anesthesia with halothane (0.5%), had a central venous catheter inserted under aseptic conditions and was submitted to a controlled hemorrhage of 1030mL (30% of total blood volume). Whole blood was collected in double bags with citrate phosphate dextrose adenine-1 and without leukoreduction filters (Fresenius Hemo Care, São Paulo, Brazil). After an infusion of 3000mL of normal saline to replace blood loss, the anesthesia was discontinued and the animal was sent to recovery. The units containing whole blood were centrifuged at 3300rpm for 16 minutes (centrifuge Beckman-Spinchron 15, Beckman Coulter, California, USA), the plasma was discarded and the two RBC units recovered were maintained at a controlled temperature (2°-6° Celsius) for 14 days. The target temperature was tracked by a temperature recorder (Ibuttom DS1921g, Maxim-Dallas, California, USA).

The validation of RBC survival and the evaluation of hemolysis in vivo were performed by labeling the RBC with radioactive $Na_2^{51}CrO_4$ and recovering the viable RBC 24 hours after transfusion, according to a method adapted from previous reports.[9,10] For these experiments, five Agroceres® male pigs (including the animal submitted to hemorrhage 14 days before) were maintained under anesthesia with halothane (0.5%) and muscle paralysis with pancuronium (intermittent infusion of 0.1mg/kg). Then, 0.7mL radioactive sodium chromate (lot 803 C 20002 IPEN/CNEN/SP; calibrated on 01/23/08, with a period of validity up to 04/11/08; 185MBq activity) was added to 100g of RBC from one of the stored RBC units. This procedure was carried out with the addition of ascorbic acid (200mg). We injected the animals with the equivalent of 2.5mL/kg (0.412MBq/kg or 0.01mCi/kgl) from the sample labeled with sodium chromate - 16mL in the four homologous animals (weight 37-38kg) and 24mL in the autologous animal (weight 60kg). Measurements were performed with ten microliters of whole blood collected at different intervals (5 min, 10 min, 1 hour, 3 hours, 6 hours, 9 hours, 12 hours, and 24 hours) according to a modified "early time values average" method.[10] An average of early counts (5 and 10 min) was performed to obtain a 100% baseline (time zero). The samples were counted in an Automatic Gamma Counter (Wizard 3 Perkin Elmer, Massachusetts, USA). The coefficients of variation were 0.3%.

The analysis of RBC survival at 24 hours was assessed by calculating the percentage of surviving RBC labeled with radioactive material up to 24 hours after the transfusion.[8] This was determined as follows:

$$24 \text{ hours RBC survival(\%)} = \frac{\text{mean net cpm/ml RBC (time = 24 h x 100)}}{\text{cpm/ml RBC (time = 0 h)}}$$

where cpm indicates counts per minute of the radioactivity in the samples during pre-fixed periods, corrected by hemoglobin and hematocrit concentrations. Time "zero" is the average between 5-10 minutes (time for homogenization of chromate in the intravascular space).

In addition, the control of free radioactivity in the supernatant of all collected samples was performed using the same methodology as described above.

One animal died in the sixth hour of the experiment (most likely due to hypoxia), and a splenectomy was carried out immediately after death. On the remaining four animals, the surgery was performed 24 hours after the completion of the experiment, after the animals were

sacrificed with a potassium chloride overdose. The spleens were examined for the identification of splenic erythrocyte sequestration with scintillography using a gamma camera (Siemens Orbiter, Hoffman Estates, Illinois, USA) with energy collimation of 360keV, phtotype centered at 320keV, and a 20% window with digital image processing.

In vitro blood validation

The validation of the packed RBC in vitro was performed on sixteen RBC units collected and stored as described above in the following experiments. The measurements were carried out on the day of collection (baseline) and on the 14th day of storage by assessing the units' volume, hemoglobin concentration and hematocrit, free hemoglobin concentration and determination of the hemolysis index through the peroxidase method, as previously described.[10] Bacterial contamination of the units was evaluated by collecting 8mL from the first two RBC units for blood culture (BacT Alert, BioMérieux, Durham, NC, USA).

Statistical analysis

Data were considered normal according to the Kolmogorov-Smirnov goodness-of-fit model and are shown as the mean and standard deviation. Data were compared with the paired *t*-test and ANOVA for repeated measures as indicated, and a p≤0.05 was considered significant. The commercially available SigmaStat 2.0 statistical package (Systat Software, California, USA) was used.

RESULTS

In table 1, we demonstrate the main characteristics of the RBC units and the results of the in vitro validation experiments. In vitro validation was carried out by the measurement of different parameters on the day of collection (baseline) and on the 14th day of storage. As expected, free hemoglobin increased significantly after storage, as well as the hemolysis index. No other significant differences in the parameters that were measured were found.

Figure 1 shows the results of the in vivo validation experiments, which included marking RBC with chromate and measuring the percentage of the cells that

remained viable up to 24 hours after infusion. There were no significant changes in the number of viable cells during this time and, after 24 hours, a mean percentage of 97.5% of the marked cells were still viable.
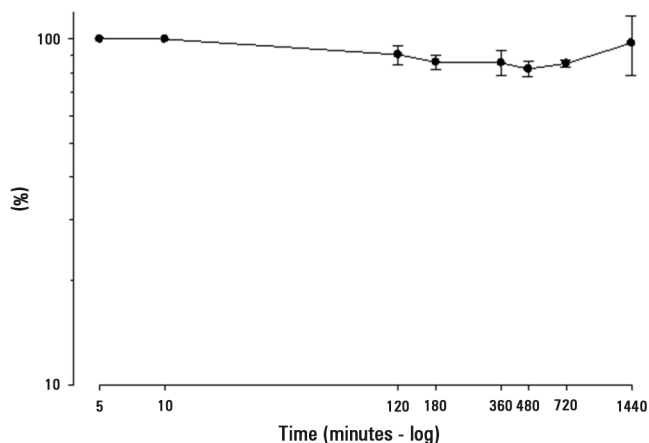

Figure 1 - In vivo viability of red blood cells marked with radioactive sodium chromate up to 24 hours.

The percentage of free radioactivity in supernatant samples at 5 min, 10 min, 120 min, 180 min, 360 min, 720 min and 1440 min was 1.1±0.1%, 1.1±0.2%, 0.9±0.2%, 0.9±0.2%, 0.7±0.1%, 1.3±1.1% and 0.8±0.3%, respectively (non-significant differences, repeated-measures ANOVA). These results ruled out significant intravascular hemolysis. In addition, spleen scintillography did not find any signs of radioactivity in the spleens, thus ruling out splenic sequestration of erythrocytes. Finally, the results of the blood culture performed on the units analyzed were negative.

DISCUSSION

The swine model has been consistently used for experimental studies of organ and tissue transplantation, as well as for intensive care medicine investigations due to its similarity to humans regarding anatomy and physiology. However, there are few experimental mechanistic studies of RBC transfusion, mainly due to difficulties in establishing animal models that resemble human conditions.[7] We aimed to develop an animal

Table 1 - In vitro validation of red blood cell units

Period	Volume (mL)	Hematocrit (%)	Hemoglobin (g/dL)	Total hemoglobin (g)	Free hemoglobin (mg/dL)	Hemolysis index (%)
Baseline	249.3±44.0	73.3±3.5	23.3±1.4	55.8±6.6	31.0±9.3	0.1±0.1
14th day storage	235.1±22.5	71.1±2.3	22.2±1.5	52.2±6.2	112.4±31.4*	0.5±0.1*

* p<0.001 versus baseline (paired *t*-test).

model of RBC transfusion in the intensive care setting. As such, our intention with the experiments described herein was to investigate if swine RBC, stored under standard blood bank conditions for 14 days, maintained their viability and therefore could be used in future experiments of transfusion related to critical illness. We demonstrated that these cells remained viable in vivo for up to 24 hours after administration and in vitro validation demonstrated the absence of significant hemolysis after 14 days of storage. Taken together, our results reinforce the possibility of developing swine experimental models to study the acute effects of transfusion in the critical care setting, thus removing several of the biases associated with transfusion studies in clinical settings.

The in vitro validation found a lower volume of the RBC units compared to humans, but the units had an equivalent total concentration of hemoglobin and hematocrit. These results are in accordance with other studies[7,11,12] and are most likely due to the lower concentrations of hemoglobin in these animals compared to humans.[7,12-14] Our results also showed a significant increase in the free hemoglobin concentration and hemolysis index after storage. However, all of the parameters investigated were in acceptable ranges using human RBC storage data as standard.[15,16] These parameters are in agreement with two previous studies from the same group that demonstrated similar alterations after storage of swine RBC for 35 days under human conditions.[7,12] Unfortunately, unlike ours, this study did not report in vivo validation of their units.

The methods used to evaluate viability of RBC in vitro are commonly not able to predict whether the sample will survive after transfusion. Thus, to evaluate the viability of stored RBC in vivo, we labeled it with a radioactive isotope and observed the percentage of RBC that remained viable 24 hours after the infusion in five animals. We demonstrated a mean percentage of 97.5% of marked cells still viable after 24 hours. These data demonstrate the adequate survival of RBC during this period, based on the gold standard for RBC viability of 75% survival of injected labeled cells at 24 hours.[17-19] Other results

that reinforce the lack of significant hemolysis are the low radioactive counts of the supernatant samples and the lack of radioactivity in the spleens of the animals, thus suggesting that the vast majority of the erythrocytes were circulating in intact form 24 hours after the transfusion.

The strengths of this study include the use of methods to measure the feasibility of the transfusion in vitro and in vivo, which has not been reported in previous studies and adds reliability to our findings. On the other hand, major limitations are the small number of animals analyzed, which could cause a type II error, and the lack of measurement of more specific markers for in vitro viability that are commonly related to storage injury, such as adenosine triphosphate, 2,3-diphosphoglycerate and lactate. In addition, we did not analyze the effects of transfusion in animal models during clinical scenarios that are more relevant to intensive care, such as sepsis and hemorrhagic shock. Thus, the clinical importance of the findings of this study is uncertain. Because this is a pilot study to assure that the transfused RBC are viable, we are now conducting experiments to investigate the mechanistic effects of transfusion in the critical care environment.

CONCLUSION

This pilot study demonstrates that swine red blood cells stored under human standard conditions for 14 days have conserved viability as evaluated in vitro using free hemoglobin levels and the hemolysis index and in vivo using chromate labeling. Our results open possibilities that could lead to experimental mechanistic studies to evaluate the effects of stored red blood cell transfusions in controlled conditions of critical illness, such as trauma or sepsis.

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RESUMO

Objetivo: Para desenvolver modelos experimentais de transfusão de hemácias, o primeiro passo é assegurar a viabilidade dos eritrócitos transfundidos. Avaliamos a viabilidade de eritrócitos transfundidos com validação *in vitro* e *in vivo* de eritrócitos suínos homólogos armazenados por 14 dias.

Métodos: Neste estudo piloto, o sangue coletado de um suíno Agroceres® foi estocado em duas unidades de hemácias. A validação *in vivo* foi realizada pela marcação dos eritrócitos com $Na_2{}^{51}CrO_4$ e recuperação dos eritrócitos viáveis após 24 horas da infusão em um animal autólogo e quatro homólogos. A validação *in vitro* foi realizada na avaliação basal e após 14 dias, pela mensuração da hemoglobina, hematócrito, índice de hemólise e

hemoglobina livre em seis unidades de hemácias. Foi realizada uma esplenectomia *post-mortem* para avaliar o sequestro esplênico de eritrócitos, e a radioatividade das amostras de sobrenadante foi contada para avaliar a hemólise intravascular.

Resultados: Após 14 dias de estocagem, as unidades de hemácias tinham volumes menores e concentração total de hemoglobina equivalente em comparação aos padrões humanos. A concentração de hemoglobina livre aumentou de 31,0±9,3 para 112,4±31,4mg/dL (p<0,001) e o índice de hemólise aumentou de 0,1±0,1 para 0,5±0,1% (p<0,001). Entretanto, esses testes se encontravam dentro da faixa aceitável para os padrões humanos.

A percentagem de radioatividade nas amostras de sobrenadante foi similar na avaliação basal e após 24 horas, afastando, assim, a presença de hemólise significante. Não se encontraram evidências de sequestro esplênico de eritrócitos radioativos.

Conclusão: Hemácias suínas estocadas por 14 dias são viáveis e podem ser utilizadas em estudos experimentais de transfusão. Esses experimentos de validação são importantes para ajudar os investigadores a estabelecerem modelos experimentais de transfusão.

Descritores: Medicina transfusional; Eritrócitos; *In vitro*; Suínos; Estudos de validação

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