Rapid CO breath test screening of drugs for protective effects on ribavirin-induced hemolysis in a rabbit model: a pilot study

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Abstract

Hemolytic anemia is a major side effect of ribavirin antiviral treatment for chronic hepatitis C. Ribavirin dose reduction may compromise the antiviral response and erythropoietin can take several weeks to alleviate anemia. The purpose of the present study was to screen potentially protective drugs against ribavirin-induced hemolytic anemia in a rabbit model, using our modified CO breath test for measuring erythrocyte lifespan (RBC), the gold standard diagnostic index of hemolysis. Fifteen rabbits were divided randomly into five groups (N = 3/group): one vehicle control group, one ribavirin (only)-treated (RBV) group, and three groups initially treated with ribavirin only, followed by a combination of ribavirin with prednisone (RBV + Pred), polyene phosphatidyl choline (RBV + PPC), or reduced glutathione (RBV + GSH). RBC lifespan was calculated from accumulated CO measured in a closed rebreath apparatus, blood volume measured by the Evan’s blue dye (EBD) dilution test, and hemoglobin concentration data. The RBC lifespan was normal in the vehicle control group (44–60 d), but reduced significantly in all of the ribavirin-treated groups before the addition of screened drugs (17–35 d). RBC lifespan rebounded significantly with the addition of glutathione, but not with the addition of prednisone or polyene phosphatidyl choline. A similar overall drug effect pattern was seen in the hemoglobin concentration and reticulocyte count data. In conclusion, the results of this pilot study indicate that reduced glutathione can attenuate ribavirin-induced hemolytic anemia, and that the RBC lifespan measured with our modified rapid CO breath test is feasible and reliable for use in animal studies.

1. Introduction

Combination drug therapies, such as the combination of the powerful antiviral agent ribavirin with the immunomodulatory agent interferon α, have become the standard approaches to treating hepatitis C virus infection. Ribavirin, a water-soluble synthetic analogue of guanosine, exerts antiviral activity by inhibiting intracellular phosphorylation reactions [1]. While this inhibition is not deleterious to most human cells, erythrocytes (RBCs) are adversely affected by it, and the vast majority of subjects receiving ribavirin develop hemolytic anemia [1, 2].

To reduce or prevent ribavirin-induced hemolytic anemia, patients are often given supplementary erythropoietin or may have their antiviral dosage reduced. While both approaches may be effective, each has disadvantages. Erythropoietin treatment is costly and takes several weeks to produce effects. Meanwhile, antiviral dosage reduction risks compromising the therapeutic response. The anti-anemia efficacy of several other compounds, such as eicosapentaenoic acid, pentoxifylline, high-dose vitamin C, and high-dose vitamin E, have been examined in clinical studies [3, 4]. However, the results have been controversial, leaving erythropoietin as the only empirically...
accepted intervention for anemia secondary to anti-viral treatment.

Because of concerns related to patient safety, cost, and the challenges of multifactor controls in clinical trials, candidate drugs are tested in vitro first and then in animal studies before being advanced for clinical trials in human patients. For example, using a simple in vitro hemolytic test, Brochot et al [5, 6] have identified several agents with a definitive protective effect against ribavirin-induced hemolysis. These agents merit further testing in animal models.

Duration of RBC lifespan is the gold standard index of in vivo hemolysis and, as such, a desirable objective measure of anti-anemia efficacy in animal studies. However, classical techniques for measuring RBC lifespan, such as $^{51}$Cr or biotin labeling protocols, are complex and time-consuming. We have developed a simple, rapid test that utilizes breath carbon monoxide (CO) concentration to quantify RBC survival in rabbits [7]. The present study is a proof of principle pilot study in which we examine the utility of using our small-animal CO breath test procedure for screening of drugs with potential efficacy for countering ribavirin-induced anemia in rabbits.

2. Materials and methods

2.1. Animals
Fifteen adult New Zealand white rabbits (2.2–2.7 kg) were purchased from Guangdong Medical Laboratory Animal Center. All rabbits were housed in a standard vivarium at Shenzhen Medical Devices Testing Center with free access to food and water. The research protocol was approved by the Laboratory Animal Ethics Committee of the Shenzhen Institute for Drug Control.

2.2. Drugs and reagents
Ribavirin (Batch number: 131201) was purchased from Sichuan Baili Pharmaceutical Co. Ltd, China. Prednisone (Batch number: 1406031) was purchased from Tianjin Pharmaceutical Co. Ltd, China. Polyene phosphatidyl choline (Batch number: 4JD108, electronic supervision code: 81232 15024 73414 22203) was obtained from Sanofi (Beijing) Pharmaceuticals Co. Ltd. Reduced glutathione (Batch number: 14040220, electronic supervision code: 81585 45004 22188 13916) was obtained from YaoPharma, China. All other chemicals and reagents were purchased from native suppliers.

2.3. Experimental design and treatment groups
The rabbits were divided randomly into five groups (N = 3/group), including one vehicle control group and four ribavirin-treated groups. The rabbits in the vehicle control group were fed 10 ml of 5% glucose (vehicle) solution daily. The rabbits in the ribavirin-treated groups were fed ribavirin (20.0 mg kg$^{-1}$ d$^{-1}$) dissolved in 10 ml of 5% glucose solution. On day 21, blood tests (described below) and the first CO breath test were conducted. Our previous research has shown that ribavirin-induced changes in hemoglobin becoming stable after 3 weeks. In the following 2 weeks, the daily ribavirin treatment continued alone or in combination with another agent. Rabbits in the ribavirin alone group served as hemolytic anemia controls. The remaining groups received ribavirin combined with 4.6 mg kg$^{-1}$ d$^{-1}$ prednisone (RBV + Pred group), 52.4 mg kg$^{-1}$ d$^{-1}$ polyene phosphatidyl choline (RBV + PPC group), or 46 mg kg$^{-1}$ d$^{-1}$ reduced glutathione (RBV + GSH group). Repeat blood tests and CO breath tests were conducted on 7 d and 14 d after the initial CO breath test, respectively.

2.4. CO breath test
As shown in figure 1, the rebreath apparatus (constructed in our laboratory) consists of a 90 l sealed bio-cage with ventilation accessories. In preparation for each test trial, the bottom of the cage was lined with soda lime (500 g; replaced every 7 h), a CO$_2$ absorbent material, and the cage was sealed closed. A 1.5 l baseline air sample was collected from the cage by pumping the cage air into an aluminum bag through a gas circuit connector. Each rabbit was placed individually into the cage after being weighed. The air circulation pump was switched...
on and then the cage was resealed. Air flowed through the cage from top to bottom at a rate of 8.0 l min⁻¹. The O₂ tension level was maintained at 20–23% by adjusting the oxygen supply flow (~4 ml min⁻¹). While the O₂ was supplemented as it was consumed, exhaled CO₂ was absorbed. Another 1.5 l gas sample was collected from the cage following 120 min of rebreath accumulation.

CO concentrations (ppm) in the air samples were analyzed by infra-red spectroscopy (RBCS-01, Seekya Biotec. Shenzhen, China). The difference between the baseline control and experimental samples was taken as the accumulated concentration of endogenous CO (P_CO). RBC lifespan (in days) was calculated based on CO measurements from the following formula, which equates mean RBC lifespan to the total capacity of CO from hemoglobin divided by the CO quantity released per day.

\[
\text{RBC lifespan} = \frac{V_b \times \text{Hb} \times 4 \times 22.4}{64400} \left( \frac{V_{\text{cage}} \times P_CO \times 10^{-0.6} \times 0.7 \times 24}{t} \right) \]

(1)

where V_b is blood volume in l (see section 2.5) and Hb is hemoglobin concentration in g l⁻¹. The main numerator (equation in the first set of parentheses) gives the total capacity of CO from hemoglobin as the product of V_b, Hb, the CO to hemoglobin molar ratio (i.e. 4), and the volume in l (i.e. 22.4) consumed by 1 mole of CO divided by the molecular weight of hemoglobin (i.e. 64 400). The main denominator (equation in the first set of parentheses) gives the daily CO quantity released as the product the cage volume \( V_{\text{cage}} \), \( P_CO \) a ppm-to-l conversion factor (i.e. \( 10^{-0.6} \)), the approximate fraction of production of endogenous CO derived from hemoglobin turnover (i.e. 0.7), the number of hours in a day (i.e. 24), and the number of hours per day divided by the rebreath time in hours \( t \). Given that \( V_{\text{cage}} \) was 90 l and \( t \) was 2.0 h in our experiment, the formula was simplified to the following expression

\[
\text{RBC lifespan} = \frac{V_b \times \text{Hb} \times 1.84}{P_CO} \]

(2)

2.5. Evan’s blue dye (EBD) dilution test

Blood volume was estimated with the EBD dilution test conducted on the same day as the CO breath test, after breath sampling. The EBD dilution test was performed as described elsewhere with slight modifications [8, 9]. Briefly, for each animal, an initial (blank) 1 ml blood sample was withdrawn from the auricular artery and 1 ml of 0.2% EBD solution in normal saline was injected into the marginal vein. Subsequently, 0.5 ml of saline was injected to wash out the injected dye. After 8 min, another 1 ml blood sample was withdrawn. All samples were collected in heparinized tubes and plasma was separated from whole blood. EBD content was determined by a spectrophotometric assay, wherein a 0.2 ml aliquot of plasma was placed in a 96-well microplate and the absorbance was detected at 650 nm with a microplate reader (SpectraMax M3, Molecular Devices, USA). Plasma volume was calculated based on the dilution principle (plasma volume = volume of injected EBD × concentration of injected EBD/concentration of EBD of measured sample). Finally, blood volume was estimated by dividing the plasma volume by difference between 1.0 and the hematocrit level.

2.6. Blood tests

The rabbits were subjected to baseline and follow-up routine blood tests. The blood samples were obtained from the marginal ear vessels. Hemoglobin concentration, hematocrit level, and reticulocyte count were determined by standard methods with an automatic hematology analyzer (Sysmex XE-2100, Japan).

2.7. Statistical analysis

Our RBC lifespan, hemoglobin concentration, and reticulocyte data are reported as means ± standard deviation (SD). The statistical analyses were performed automatically in SPSS20.0. Repeated-measures analyses of variance (ANOVA) were used to examine mean differences in relation to independent variables, including time point and treatment condition.

3. Results

All 15 rabbits finished the study as planned. The RBC lifespan data measured by the CO breath test in individual rabbits are reported in table 1 and the mean values for each group at each time point are shown in figure 2. The RBC lifespans of rabbits with 21 d of ribavirin treatment were shorter than those of vehicle control animals. Additionally, the ribavirin-treated groups showed a
significant decrease in hemoglobin concentration and a significant increase in reticulocyte count (table 2). RBC lifespans, hemoglobin concentrations, and reticulocyte counts of the vehicle group rabbits stayed within a normal range throughout the study period [10–12]. Among the combination treatment groups, only the RBV + GSH group exhibited a significant recovery of RBC lifespan, hemoglobin concentration, and reticulocyte count relative to the RBV only group. The RBC lifespan recovery effect was relatively rapid, reaching control levels within a week, whereas the hemoglobin and reticulocyte recoveries remained partial after 2 weeks.

4. Discussion

In this pilot study, we screened putative hemolysis-protective drugs in rabbits using our CO breath test method of RBC lifespan measurement. Repeated measures of RBC lifespan of the vehicle control rabbits remained within the normal range throughout the whole study period, while the RBC lifespans of rabbits given ribavirin treatment courses were reduced significantly. The ribavirin effects on RBC lifespan were accompanied by significant decreases in hemoglobin concentration and significant reticulocyte count increases. These results provide a demonstration of a rabbit model of ribavirin-induced hemolytic anemia and also demonstrate the feasibility and reliability of our small-animal CO breath test method of measuring RBC lifespan, which is the gold standard index of hemolysis.

Among the three tested drugs, only reduced glutathione showed a reversal effect on ribavirin-induced hemolysis, producing significant recoveries of RBC lifespan, hemoglobin concentration, and reticulocyte count, while the other two tested drugs, namely prednisone and polyene phosphatidyl choline, did not affect any of the measured parameters. Prednisone is used to

Table 2. GSH treatment results in partial amelioration of RBV-associated changes in hemoglobin concentration and reticulocyte count (mean ± SD).

| Group       | Hemoglobin (g l\(^{-1}\)) | Reticulocyte count (%) |
|-------------|---------------------------|------------------------|
|             | Day 0  | Day 7  | Day 14 | Day 0  | Day 7  | Day 14 |
| Vehicle     | 131 ± 8.6 | 135 ± 7.2 | 125 ± 3.7 | 2.4 ± 0.1 | 2.3 ± 0.2 | 2.7 ± 0.4 |
| RBV         | 96 ± 3.8* | 96 ± 5.5 | 102 ± 7.1 | 6.1 ± 1.5* | 4.1 ± 0.5 | 4.9 ± 0.2 |
| RBV + Pred  | 78 ± 9.2* | 81 ± 10.8 | 81 ± 10.8 | 11.2 ± 0.8* | 7.8 ± 1.0 | 8.9 ± 3.4 |
| RBV + PPC   | 92 ± 5.0* | 94 ± 7.2 | 84 ± 12.7 | 6.1 ± 0.9* | 5.0 ± 0.9 | 8.3 ± 0.9 |
| RBV + GSH   | 89 ± 8.5* | 87 ± 12.0 | 99 ± 6.0\(^{0.00}\) | 9.3 ± 2.2\(^{0.00}\) | 10.8 ± 2.0 | 7.0 ± 2.3\(^{0.00}\) |

\(^{0.00}\)P < 0.01 versus vehicle; hemoglobin day 0, \(F_{(1,10)} = 2376.521\); reticulocyte day 0, \(F_{(1,10)} = 557.337\).

Abbreviations. RBV, ribavirin; Pred, prednisone; PPC, polyene phosphatidyl choline; GSH, reduced glutathione.
treat autoimmune hemolytic anemia, whereas polyene phosphatidyl choline (a lipid extracted from soybean) is used as a cell membrane protective agent in patients with chronic liver diseases. The lack of efficacy of prednisone in this context suggests that the mechanism of ribavirin-induced hemolysis is unrelated to an autoimmune disturbance, and that polyene phosphatidyl choline did not provide adequate protection of RBC membranes.

Reduced glutathione is a very important antioxidant. Epidemiological surveys have found that erythrocyte glutathione depletion impairs resistance to hemolysis in women consuming alcohol, and that elevated erythrocyte glutathione protects against smoking induced hemolysis [13, 14]. Additionally, in vitro studies have shown that glutathione inhibits ribavirin- or radical-induced hemolysis strongly [5, 15]. Although the exact mechanism of ribavirin-induced hemolytic anemia is not known, recent studies have suggested that it may be the result of oxidative damage to the erythrocyte membrane that results in erythrophagocytosis by the reticuloendothelial system [16]. Our positive results suggest that GSH, which is already appreciated for its antioxidative qualities, should be examined as a potential therapy for ribavirin-induced hemolysis.

Our hemoglobin concentration and reticulocyte count data complemented our RBC lifespan data. However, it should be noted that although these two tests are simple to perform, their results provide only indirect parameters of hemolytic anemia. Consequently, they are sensitive to artefactual effects, such as variations due to non-hemolytic conditions such as malnutrition, hematopoietic disorders, and blood loss, etc. In contrast, RBC lifespan provides a direct assessment of hemolysis. Moreover, the present data showing that RBC lifespan recovered before hemoglobin levels or reticulocyte counts underscores the dissociation of these parameters and indicates that screening based only on these indirect parameters would not provide a reliable assessment of current anemia status.

Reduced RBC lifespan is the gold standard for hemolysis diagnosis. The classic standard methods for determining RBC lifespan are reinfusion measurements of labeled RBCs, including 51Cr-labeling developed in the 1950s and biotin-labeling developed in the 1980s [17]. The major drawbacks of these two methods are that they require multiple venesections and that these multiple procedures must be conducted over a period of several weeks, or even months. As a result, neither labeling method has become well established in animal research or clinical practice. CO is a catabolic byproduct of heme that originates from the stoichiometric conversion of the α-methene carbon of the porphyrin ring to CO during the catabolism of heme to bilirubin. Because hemoglobin breakdown represents the majority (70–80%) of heme turnover, production of CO reflects RBC turnover closely [18]. In 1966, using a complicated rebreath system with continuous monitoring of blood carboxyhemoglobin (COHb) concentrations, Coburn et al [19] demonstrated that mean erythrocyte lifespan can be considered to be equal to the total CO capacity from degradation of all hemoglobin divided by the amount produced per day. Strocchi et al [20] developed a straightforward, noninvasive CO breath test to estimate RBC lifespan in human subjects, which was later simplified by Fume et al [21]. The method was confirmed to have good consistency with RBC survival status [22–26].

Strocchi’s protocol, however, is not practical for use with small animals, for whom collection of alveolar breath samples would be very difficult. To overcome this challenge, we developed a simple apparatus for accumulated CO collection based on a rebreath mechanism (figure 1). Our method measures expired endogenous CO that has been allowed to accumulate in a sealed box, removing the need for continuous monitoring of blood COHb concentrations [19]. We have demonstrated previously that RBC lifespan data calculated based on accumulated CO concentration measured with our rebreath system, blood volume determined by the EBD dilution technique, and hemoglobin concentration were virtually identical to RBC lifespan data obtained by the biotin labeling method [7]. The present study provides further demonstration of the reliability of this method. In practice, operation of the rebreath apparatus is very simple and the data are measured in an automated fashion. Furthermore, the rebreath system enables RBC lifespan to be determined within a few hours rather than in several weeks as is needed with the currently available RBC labeling approaches [7].

5. Conclusion

Using RBC lifespan as a judgment criterion, this study suggests that reduced glutathione may provide protection against ribavirin-induced hemolytic anemia and thus merits further study and clinical trials. Additionally, the present work demonstrated that our CO breath test method is a feasible and reliable means of determining RBC lifespan in animal studies. Its use may facilitate the screening of candidate drugs for the treatment and prevention of hemolytic anemia.

Conflict of interest

The authors have no conflict of interest to declare.

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