Journal of Pharmacological and Toxicological Methods 84 (2017) 44–50

Contents lists available at ScienceDirect

Journal of Pharmacological and Toxicological Methods

journal homepage: www.elsevier.com/locate/jpharmtox

Research article

A new rat model of neonatal bilirubin encephalopathy (kernicterus)

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ARTICLE INFO

Article history:
Received 4 April 2016
Received in revised form 3 October 2016
Accepted 9 October 2016
Available online 13 October 2016

Chemical compounds studied in this article:
Sulfisoxazole (PubChem CID: 5344)
Phenyl hydrazine hydrochloride (PubChem CID: 75331)
Nitric acid (PubChem CID: 944)
Methanol (PubChem CID: 887)
Sucrose (PubChem CID: 5988)
Tris (PubChem CID: 6503)
Hydrogen peroxide (PubChem CID: 784)
Paraformaldehyde (PubChem CID: 712)
Xylitol (PubChem CID: 6912)
Chloroform (PubChem CID: 6212)

Keywords:
Kernicterus
Hyperbilirubinemia
Phenyl hydrazine
Sulfisoxazole

ABSTRACT

Introduction: Hemolytic kernicterus, an indirect bilirubin-induced brain dysfunction, is associated with hyperbilirubinemia in mammalian neonates. In this study, a new model of kernicterus has been developed using intra-peritoneal injections of phenyl hydrazine and subcutaneous injections of sulfisoxazole. These drugs can potentially induce kernicterus in neonatal through changes in hemolysis and hypo-albumin.
Methods: For this purpose, 7-day-old male Wistar rats ($n = 72$; mean weight 11 ± 1 g) were used. The animals have been divided into six different groups which received the drugs alone and their combination, and the drugs' solvents and their combination. Biochemical parameters, brain iron and bilirubin, behavioural performance, auditory function and apoptosis were measured using auto-analyser instruments; atomic absorption spectroscopy, Sawasaki, footprint, auditory brainstem response (ABR) and TUNEL test, respectively.
Result: The drug-injected groups showed a significant reduction in serum haematocrit and an increase in the concentration of brain bilirubin, total and indirect bilirubin as well as TUNEL positive cells in basal ganglia. In addition, the obtained results showed that there was a significant increase in behavioural disturbance and auditory dysfunction in the group injected with the combination of two drugs.
Conclusion: This kernicterus-induced rat model could perfectly mimic the common conditions of the hyperbilirubinemia in human neonates. This study offers an easy technique to develop more stable models for follow-up studies.

1. Introduction

Kernicterus, a bilirubin-induced brain dysfunction, is associated with hyperbilirubinemia in mammalian neonates. Accumulation of indirect bilirubin (IB)[1] in brain regions particularly the basal ganglia, cerebellum, brain stem nuclei, and cochlear nucleus causes irreversible neurological damages in neonates (Shapiro, 2003). The clinical features of kernicterus include neurological impairments such as motor-development delay, hearing loss, epilepsy, cerebral palsy, mental retardation, lethargy, and poor nutrition. Since no specific therapeutic strategy exists

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[1] IB (indirect bilirubin): indirect or non-conjugated bilirubin that cannot be excreted from the blood and according to previous studies, increase of this factor in the blood is the main cause of kernicterus.

http://dx.doi.org/10.1016/j.vascn.2016.10.002
1056-8719/

for these patients, kernicterus may lead to death or permanent nervous system complications (Pediatrics, 2004).

As a by-product of heme catabolism, bilirubin is derived from heme via two enzymatic processes involving hemoxygenase and biliverdin reductase (Kikuchi, Yoshida, & Noguchi, 2005; Shapiro, 2002). When the rate of bilirubin production exceeds the body's capacity to conjugate it with serum albumin, serum total bilirubin (TB)[2] is increased and hyperbilirubinemia may occur (Stevenson, Dennery, & Hintz, 2001). It has been shown that elevation of bilirubin concentration in blood by administration of exogenous bilirubin or sulfonamide drugs such as sulfisoxazole and sulfadimethoxine results in the augmentation of bilirubin concentration in the brain and occurrence of kernicterus in neonates (Cetin et al., 2009; Ostrow, Pascolo, Shapiro, & Tiribelli, 2003). Sulfonamides displace bilirubin from albumin binding sites in plasma and consequently elevate plasma level of bilirubin which then crosses the blood–brain-barrier and enters the central nervous system to induce kernicterus (Thyagarajan & Deshpande, 2014).

Various kernicterus models have been developed in animals to study the neurophysiology of the disease and also the effectiveness of different therapeutic approaches. Induction of Crigler–Najjar syndrome is an approach to generate a congenital animal model called “Gunn rats” through the enhancement of free bilirubin concentration which leads to the development of jaundice in these animals. In another model, reaction of a compound called phenyl hydrazine with the carbonyl group of various biologically remarkable molecules, along with its interactions with haemoglobin and cytochrome P450 causes the development of free radicals. These free radicals then become involved in haemolysis (Mejia et al., 2008). Although all animal models of kernicterus are valuable for investigation of the underlying physiological mechanisms of this disorder and confirming the toxic effects of bilirubin accumulation in nervous system, inconsistent results are usually observed when comparing animal and human studies. This is due to the lack of an appropriate animal model similar to human complication. Therefore, a simple and affordable animal model of haemolytic kernicterus is needed that closely mimics the clinical manifestations of haemolytic kernicterus in human new-borns.

According to the previous studies, the rat brain at 7th day of development is histologically similar to human preterm infant at 32 to 34 weeks of gestation (Mejia et al., 2008). In the present study, we have created a new model of kernicterus using an intra-peritoneal injection of phenyl hydrazine into 7-day-old new-born rats followed by subcutaneous injection of sulfisoxazole at 9th day. Thus, by using phenyl hydrazine to induce haemolysis (Mejia et al., 2008) and sulfisoxazole to displace bilirubin from albumin binding sites (Hansen, 2002), our method was successful in generating an animal kernicterus model similar to human neonates.

2. Materials and methods

2.1. Animal study design

In this research, the experimental protocol for animal study was approved by the Animal Research Committee of Iran University of Medical Sciences (protocol number: 24,317) and complied with our Institutional and National Guide Rules for Care and Use of Laboratory Animals. To provide animal models, 7-day-old male Wistar rats ($n = 72$; mean weight 11 ± 1 g) were used. The rats were housed at 21–23 °C with controlled humidity ($50\% \pm 5\%$) and 12 h artificial light cycles (7 am–7 pm). At the beginning of the experiment, the rats were randomly divided into six experimental groups, in regard to phenyl hydrazine (P) and sulfisoxazole (S) exposure: P, Control P, S, Control S, P + S and Control P + S. In group P, haemolysis was induced by the intraperitoneal administration of 50 mg/kg of phenyl hydrazine hydrochloride (Rice & Shapiro, 2008) (Sigma Aldrich, USA) in 7-day-old rats. In group Control P, the animals received phenyl hydrazine solution in normal saline (0.1 ml normal saline 0.9%). To induce blockade of bilirubin binding sites, 250 mg/kg of sulfisoxazole (Rice & Shapiro, 2008) (Abcam, USA) was injected subcutaneously into 9-day-old rats in group S. In group Control S, 9-day-old rats received sulfisoxazole solution in DMSO (0.09 ml DMSO). In group Control P + S, the animals received normal saline and DMSO. The last group received phenyl hydrazine and sulfisoxazole. In this group, 50 mg/kg of phenyl hydrazine was injected intra-peritoneal into 7-day-old rats, and 250 mg/kg of sulfisoxazole solution administered subcutaneously in the same rats 2 days later. Since normal saline is the solvent for Phenyl hydrazine, it was injected to group Control P. Moreover, DMSO is the solvent for sulfisoxazole that injected into group Control S. The following diagram (Diagram 1) has been drawn for better understanding of the presented groups, total number of animals in each group and timing of each procedure. In total, 24 h after the injection of phenyl hydrazine and 2 h after the injection of sulfisoxazole (Rice & Shapiro, 2008), the levels of total, direct and indirect bilirubin and haematocrit were measured ($n = 6$). Then, the rats were sacrificed with CO_2 inhalation, and the levels of brain bilirubin (BB)[3] and iron were measured in the animals.

In total, 24 h after achieving the highest peak of the drug, the Auditory Brainstem Response (ABR) test was performed to confirm the generation of kernicterus model ($n = 6$). Footprint and Tunnel tests were also performed for analysing walking and cell apoptosis, respectively ($n = 6$). In animals that received a combination of phenyl hydrazine and sulfisoxazole, the levels of bilirubin and iron were investigated after the injection of sulfisoxazole at day 9. The ABR, footprint and Tunnel tests were carried out again, 24 h later, on day 10. According to Schenker, David, Mccandless, and Paul (1966), sulfisoxazole had greater effects at 2 h post-injection; while Rice and Shapiro (2008) reported similar results for phenyl hydrazine at 24 h post-injection.

2.2. Compounds

Sulfisoxazole (Abcam, USA) was dissolved in DMSO (0.09 ml). Phenyl hydrazine hydrochloride (Sigma Aldrich, USA) was dissolved in normal saline (0.1 ml normal saline 0.9%). Nitric acid (Merch, Germany) 1:10 wt/vol dilution. Methanol (Merch, Germany), Tris (Sigma Aldrich, USA), Hydrogen peroxide (Sigma Aldrich, USA), Paraformaldehyde (Sigma Aldrich, USA), Xylitol (Sigma Aldrich, USA) and Chloroform (Sigma Aldrich, USA) were dissolved in deionized water.

2.3. Measurement of biochemical and histological parameters

To explore the variety of bilirubin level, blood samples were collected from animals' hearts. In addition, in order to evaluate haematocrit, blood samples were collected from the corner of eyes. After separation of serum using centrifuges, the amount of bilirubin were measured by auto analyser. The brain was extracted and fixed in paraffin and serum samples were frozen at −70 °C. The haematocrit levels, as an indicator of haemolysis, were determined by counting red blood cells. The measurements of total and direct bilirubin (DB)[4] were carried out using an auto-analyser setting. Total serum bilirubin higher than 3 mg/dl was considered as an indicator of hyperbilirubinemia (Cruz, 2008; Dominguez Ortega, Gonzalez Azpeitia, Cidras Pidre, & Calvo Rosales, 1997).

For the measurement of brain bilirubin, the brain bilirubin levels were measured according to Sawasaki's method (Meier et al., 2006). To do that, the brain tissue was homogenized in 0.25 M sucrose solution (1:3 v/v). Then, bilirubin was extracted using a mixed solution

[2] TB (total bilirubin): summation of direct and indirect bilirubin in the blood that indicates decomposition of red blood cells is too much.

[3] BB (brain bilirubin): It is amount of bilirubin in brain tissue which indicates the arrival of bilirubin from the blood to the brain and damage to the brain.

[4] DB (direct bilirubin): conjugated bilirubin that easily excreted via urine and stool and is not harmful for CNS.