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Development of an experimental rat model of hyperammonemic encephalopathy and evaluation of the effects of rifaximin

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ABSTRACT

Hepatic encephalopathy (HE) is a neuropsychiatric syndrome associated with hepatic dysfunction. However, the precise mechanism of HE is unclear. To elucidate the mechanism, we developed a new rat model of HE with coma using a combination of subcutaneous splenic transposition, partial hepatectomy and portal vein stenosis. In this model, blood ammonia levels increase in the postcaval vein over time and markedly increase in the cerebrospinal fluid (CSF). The distribution of ammonia in the various blood vessels in the HE model suggests that the origin of peripheral blood and CSF ammonia is the mesenteric veins that drain blood from the gastrointestinal tract. Behavioral analysis revealed decreased pain response, increased passivity, and decreased pinna and corneal reflexes, followed by the development of coma. The development of coma in this model was frequent and reproducible. Increased S100 calcium-binding protein B (S100B: a biomarker for brain injury) in venous blood, as well as damaged brain tissue, increased intracranial pressure and cerebral edema were observed in rats with coma. A very high correlation was observed between the blood ammonia concentration in the postcaval vein and the onset of coma. Rifaximin, a poorly absorbed antibiotic that targets gut flora, significantly improved symptoms of HE. Based on these results, our rat model appears to reflect the pathological state of HE associated with acute liver failure and may be a useful model for analysis of hyperammonemic encephalopathy.

1. Introduction

Hepatic encephalopathy (HE) is an important complication of chronic and acute liver disease (Riordan and Williams, 1997; Sturgeon and Shawcross, 2014). Previously, HE has been classified into three types: Type A, HE associated with acute liver failure (ALF); type B, HE associated with portal-systemic shunting without hepatocellular disease; and type C, HE associated with underlying cirrhosis and portal hypertension or portal-systemic shunts (Ferenci et al., 2002). The pathophysiology of HE is not completely understood, although there is agreement on the important role of neurotoxins, particularly ammonia (Romero-Gómez et al., 2015). Ammonia is produced from several organs, such as kidneys and muscle, and its concentration is highest in the portal venous system in dog, sheep and horse (Hahn et al., 1893; Nencki and Zaleski, 1895; Nencki et al., 1896). In humans, portal ammonia is presumed to be derived from both the urease activity of the colonic bacteria and the deamination of glutamine in the small intestine (Romero-Gómez et al., 2015). Therefore, we examined the distribution of blood ammonia in rats, given its fundamental role in the pathology of HE.

In clinical practice, the blood ammonia level is widely used for diagnosing HE; blood levels correlate with an increased risk of encephalopathy (Bernal et al., 2007). However, the correlation between blood ammonia levels and HE severity is not consistent (Bosoi et al., 2011; Ong et al., 2003; Shawcross et al., 2011). In the absence of an appropriate animal model of HE, there may be discrepancies between experimental results and clinical signs. For example, a drug-induced hepatotoxicity model has been shown to induce definite pathological lesions, but does not reproduce the same behavioral signs observed in HE patients (Butterworth et al., 2009). Coma is an important behavioral change in HE patients and should be evaluated as a pathognomonic feature (Leise et al., 2014; Vilstrup et al., 2014). Other models, for example the portal-systemic anastomosis model, require a high degree of microsurgical skill and lack general versatility (Funovics et al., 1975; Jover et al., 2005). We thus established a new rat model of HE using an efficient surgical method that resulted in hyperammonemia and

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coma. The objectives of this study were to develop a reliable HE model and clarify the correlation between blood ammonia levels and the onset of coma. Rifaximin is an oral, nonsystemic, broad-spectrum antibiotic that targets gut flora (Bass et al., 2010) and is used clinically to reduce serum ammonia derived from the gastrointestinal (GI) tract. We thus used rifaximin to investigate the role of ammonia in our HE model.

2. Materials and methods

2.1. Animals

All study procedures were carried out after approval of the protocol by the Animal Research Committee of ASKA Pharmaceutical Co., Ltd. based on the criteria, "Rules for the Care and Use of Laboratory Animals". Male Sprague Dawley rats (Charles River Laboratories Japan, Inc., Yokohama) weighing 170–230 g were used. The animals were maintained at constant temperature (22 °C) on a 12 h light/dark cycling schedule (8:00 on and 20:00 off) with free access to food and water.

2.2. Drugs

Dosing formulations were freshly prepared on the day of use and administered orally in doses ranging from 2 to 5 ml/kg. Rifaximin was manufactured by Alfa Wassermann S.p.A., Bologna, Italy. Tween® 80 was obtained from MP Biomedicals, LLC, Santa Ana, CA.

2.3. Experimental design and surgical procedure

Following subcutaneous splenic transposition (SST), rats were orally administered either vehicle (0.5% Tween 80) or rifaximin (0.3, 3 and 30 mg/kg) once daily for 3 days. Three hours after the final dose, animals underwent 70% partial hepatectomy and portal vein stenosis to induce encephalopathy. The time of coma onset was monitored out to 24 h after HE-inducing surgery. Coma was defined as loss of the righting reflex. At the onset of coma or at 24 h after surgery, blood was collected from various vessels for determination of ammonia concentration.

2.4. Subcutaneous splenic transposition (SST)

SST was performed according to the method described by Di Domenico et al. (2007). Animals were placed in the dorsal position under isoflurane anesthesia (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and an approximately 1 cm incision was made on the skin below the left costal arch. A subcostal pouch was created laterally in the subcutaneous tissue. The spleen was decapsulized and placed into the pouch. Animals were allowed to recover for 4–6 weeks in order to allow for the development of collateral blood vessels around the spleen.

2.5. Partial hepatectomy and portal vein stenosis

The animals were placed in a dorsal position under isoflurane anesthesia and the abdomen was incised to remove 3 lobes of the liver (right, left medial and left lateral), which accounted for approximately 70% of the total liver weight (Nikfarjam et al., 2004). The portal vein and a stainless steel tube (outer diameter 0.5 mm) were ligated using a surgical suture. The steel tube was then removed, leaving the vessel ligated to approximately 0.5 mm. In this manner, blood from the portal vein was shunted into the systemic circulation.

2.6. Determination of ammonia and S100 calcium-binding protein B (S100B)

The ammonia levels in blood and cerebrospinal fluid (CSF) were assayed according to the enzymatic cycling method (Yamaguchi et al., 2005). A small sample (50-100 μl) was taken from the vessel or cerebral ventricle, mixed with a deproteinizing agent (Kanto Chemical Co., Inc., Tokyo, Japan) and centrifuged. Ammonia levels in the supernatant were measured using the Ammonia Assay Kit (Kanto Chemical Co., Inc., Tokyo, Japan). Serum levels of S100B were assessed by a sandwich enzyme immunoassay (Rat S100B ELISA kit, Uscn Life Science Inc., China).

2.7. Behavioral procedures

Behavioral tests were performed by blinded researchers at 1, 6, 12 and 24 h after HE-inducing surgery. Animals were first placed in a transparent box ($345 \times 403 \times 177$ mm^3) and observed for signs of abnormal behavior. A battery of Irwin's basic neurological tests (Irwin, 1968; pain response, pinna reflex, corneal reflex, righting reflex and passivity) was then performed. Behavioral tests were scored on a scale of 0–4 (normal score $=0$, maximal score $=4$).

2.8. Histological analysis

Animals were euthanized by exsanguination under anesthesia at various time points following surgery. The brain was removed and immediately fixed in 10% neutral buffered formalin. The cerebrum including the hippocampus was trimmed. The tissues were embedded in paraffin wax and sliced into 4 μm sections. Sections were stained with hematoxylin and eosin for histopathological examination.

2.9. Intracranial pressure (ICP) monitoring

Following HE-inducing surgery, the midline skin was incised to expose the atlanto-occipital membrane. The cisterna magna was stereotaxically punctured with a 25 G needle, which was connected to a pressure transducer via PE50 tubing. The needle was confirmed to be correctly placed in the subarachnoid space when ICP immediately increased after applying abdominal pressure. The punctured site was sealed with a drop of glue to prevent CSF leakage. A syringe column containing artificial CSF (115 mM NaCl, 5 mM KCl, 1.5 mM $CaCl_2$, 1.3 mM $MgCl_2 \cdot 6H_2O$, 35 mM $NaHCO_3$, 1.25 mM NaH_2PO_4 and 10 mM glucose; all chemicals from Sigma-Aldrich, St. Louis, MO) was also connected to the transducer. During the procedure, body temperature was monitored using an intraperitoneal thermistor and maintained at 37 °C with a ventral heating pad. Under these conditions, ICP was measured continuously for 12 h.

2.10. Quantification of brain water content

At the end of the experiment, animals were euthanized by exsanguination under anesthesia and the brain was extracted. Brain tissues were weighed and placed in an electro-thermostatic baking oven at 105 °C for 48 h until a constant dry weight was obtained or until the difference between 2 consecutive dry weights was less than 0.0001 g. The brain water content of each sample was calculated according to the following formula:

$$\text{Brain water content} = (\text{Wet weight} - \text{Dry weight})/\text{Wet weight} \times 100\%$$

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