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Original Paper

Evaluation of the Safety and Adverse Effects of Goreisan/Wulingsan, a Traditional Japanese-Chinese Herbal Formulation (Kampo), in a Rat Model: a Toxicological Evaluation

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Abstract: Diarrhea is the second leading cause of death among children less than 5 years of age. Most of these deaths occur in developing countries in the tropical areas of Africa and South Asia. Goreisan/Wulingsan, a formula of Japanese-Chinese medicinal herbs (Kampo), has been used for the treatment of diarrhea and vomiting from ancient times in East Asia. Therefore, we planned a randomized controlled clinical trial of Goreisan/Wulingsan in Bangladeshi children. Although it is believed to be safe in East Asia, information regarding its toxicity on animals is scarce. Since Goreisan/Wulingsan has never been used in Bangladesh, it was necessary to ensure the safety of the formula in an animal experiment. Rats were assigned to a control group (normal saline, n = 4) or various Goreisan/Wulingsan groups (n = 26) receiving doses of 1 to 8 mg/g/day (7.7 to 61.5 times the recommended pediatric dose) over a period of 25 days. Their activities and health conditions were observed until they were sacrificed, after which blood samples were collected for biochemical liver function tests. The kidneys, liver and heart tissue were collected for histopathological study. No lethality was observed during the experiment. All of the rats consumed the doses completely and no constipation was observed, suggesting the absence of any inhibitory effect on intestinal motion. Also, no abnormal neurological activity was detected, nor any significant elevation of AST, ALT or ALP levels, except for AST and ALT at the highest dose of 8 mg/g/day. Histopathological studies of the kidneys, liver and heart tissues revealed no abnormalities.
In conclusion, our results showed that Goreisan/Wulingsan is safe for rats, thereby justifying the use of the drug in a human trial.
Key words: Kampo, toxicology, inhibition of intestinal motion, liver function, histopathology

Introduction

Acute diarrhea is the second leading cause of death among children younger than 5 years of age, with 760,000 childhood deaths and 1.7 billion cases annually throughout the world. Most of those deaths occur in developing countries in tropical areas of South Asia and Africa [1]. Acute diarrhea is frequently complicated with vomiting. Viral gastroenteritis, characterized by the acute onset of vomiting and watery diarrhea, is the most frequent cause of diarrhea in children [2]. Therefore, acute gastroenteritis is a serious health problem for children living in developing countries, especially in tropical areas. Goreisan/Wulingsan (called Goreisan in modern Japanese and Wulingsan in modern Chinese using the original ancient Chinese characters) has been used for the treatment of acute watery diarrhea and vomiting for two thousand years in East Asia, i.e. China, Korea, and Japan. It consists of five Japanese-Chinese medicinal herbs, described later in the materials and methods section. Goreisan/Wulingsan has been regar-

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ded since ancient time as a safe medicine. No severe adverse effects of Goreisan/Wulingsan have been reporeted since 1963 when the Japanese government approved the clinical use of Goreisan/Wulingsan powder in its National Health Insurance System [3]. The Goreisan/Wulingsan used in the present study was in the form of Goreisanryo extract granules (N-17) manufactured by Kotaro Pharmaceutical Co., Ltd. A database of reported adverse effects of Goreisanryo extract granules (N-17) from 1989 to 2007 shows only three cases involving mild adverse effects: two cases of abdominal pain and one case of exacerbated edema. All of these adverse effects promptly improved on their own without additional care (information from Kotaro Pharmaceutical Co., Ltd. Osaka, Japan). However, the surveillance of adverse Goreisan/Wulingsan effects has only been conducted in Japan. A Japanese manufacturer of herbal medicine (Tsumura Co., Ltd, Tokyo, Japan) reported a toxicological evaluation of their product, Goreisan Extract Granules TJ-17, using a rat model [4]. However, the herbal components of their TJ-17 product are a modified version of the original formulation of Goreisan/Wulingsan. Modifed Goreisan (TJ-17) uses Atractylodis Lanceae Rhizoma instead of Atractylodis Rhizoma. The lethal single dose of modified Goreisan (TJ-17) has been reported to be more than 12 mg/g. Repeated doses of 3 mg/g of modified Goreisan (TJ-17) over a one-month period did not induce any toxicological effect. Nevertheless, information regarding the safety of original Goreisan/Wulingsan in an animal model is scarce.

Recently, in an open label, controlled trial for children with acute vomiting, Goreisan/Wulingsan proved to be more effective at reducing vomiting than domperidone, a dopamine antagonist [5]. With these results in mind, we planned to undertake a randomized, double-blind, placebo controlled, clinical trial (RCT) to evaluate the efficacy of Goreisan/Wulingsan in reducing vomiting and diarrhea among children with acute gastroenteritis. We decided to conduct the RCT on children in Bangladesh (ISRCTN: 34440093), a country in South Asia where the morbidity and mortality of acute gastroenteritis are high [6]. However, the practice of using Goreisan/Wulingsan is new to Bangladesh. Since most Bangladeshi (South Asian) people belong to a different race than East Asian people, it is speculated that the former might be genetically less tolerant to this medicine. However, no data or previous reports are available to shed light on this issue.

Other than its curative effect on vomiting and diarrhea, Goreisan/Wulingsan is well known for its diuretic effect. Ahn et al. reported that the diuretic and natriuretic effect of Goreisan/Wulingsan may be due to an inhibition of the renin-angiotensin-aldosterone system [7]. Goreisan/Wulingsan has also been reported to reduce renal stone formation [8, 9]. Therefore, Goreisan/Wulingsan, which exerts an effect on the kidney, may adversely affect kidney function. Okada et al. reported that Goreisan/Wulingasn decreased transaminase levels in mice fed highly fatty foods and ethanol [10]. Since Goreisan/Wulingsan exerts an effect on liver function, it may also adversely affect liver function. As for modified Goreisan (TJ-17), liver dysfunction, although mild and rare, has been reported as an adverse effect in humans. Accordingly, it was deemed important to ensure the safety of this traditional Japanese-Chinese herbal medicine with an animal model before starting a clinical trial. In this study, the toxicity of Goreisan/Wulingsan was evaluated using a Long Evans rat model.

Materials and Methods

Dehydrated extract of Goreisan/Wulingsan

A dehydrated extract of Goreisan/Wulingsan, free of all excipients, was kindly supplied by the Kotaro Pharmaceutical Co., Ltd. (Osaka, Japan). A total of 3.2 g of dehydrated Goreisan/Wulingsan was obtained from a mixture of the following herbal medicines: Alismatis Rhizoma (root of *Alismaorientale* Juzepczuk) 6.0 g, Atractylodis Rhizoma (root of *Atractylodes japonica* Koidzumi ex Kitamura) 4.5 g, Polyporus Sclerotium (body of *Polyporusumbellatus* Fries) 4.5 g, Cinnamomi Cortex (bark of *Cinnamomum cassia* Blume) 2.5 g, and Poria Sclerotium (body of *Poriacocos* Wolf) 4.5 g. Cinnamic acid, Alisol A, and Atractilenolide III are major ingredients of Cinnamomi Cortex, Rhizoma Alismatis and Rhizoma Atractylodis, respectively.

Animal study

Male and female Long Evans rats (with an average weight of 165.25 g) were purchased from the Animal Resources Branch of the International Centre for Diarrheal Diseases and Research, Bangladesh (ICDDR,B). They were fed the standard pellet diet provided by the ICDDR,B and water *ad libitum*. The toxicity study was performed to determine the safety limit of Goreisan/Wulingsan in Long Evans rats and any adverse effects the drug might exert on the kidney, liver and heart tissues of the animals. It was conducted according to the Dhaka University's Guidelines for the Use of Laboratory Animals and Experiments and it was approved by Dhaka University's Ethical Committee for Animal Experimentation.

Experimental design

The rats were assigned to a control group fed standard

feed pellets and normal saline (n = 4), and Goreisan/Wulingsan groups fed standard feed pellets and medicinal doses of Goreisan/Wulingsan: 1 mg/g/day (n = 8); 2 mg/g/day (n = 6); 4 mg/g/day (n = 7); and 8 mg/g/day (n = 5). The rats received normal saline or different doses of Goreisan/Wulingsan powder for 25 consecutive days. The activity, appetite, and constipation of the rats were observed visually and recorded.

Collection of blood samples

Blood samples were collected separately from the throat vein of each rat after sacrificing the animals at the end of the experiment. The blood samples were allowed to clot at room temperature and then centrifuged at low speed for 15 minutes. The separated serum from each rat was stored at –20°C and brought to room temperature prior to analysis.

Collection of tissue samples

Collected promptly after sacrificing the rats, the kidney, liver and heart tissues were sliced into pieces of a few millimeters in thickness, immersed in properly labeled glass beakers containing 10% formaldehyde and then stored for three days.

Biochemical and histopathological analysis of the samples

Biochemical liver function tests, i.e. measurement of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in the serum of the rats, were carried out in the Laboratory Medicine Department at the Institute of Child and Mother Health (ICMH). The biochemical analyses were carried out using colorimetric spectrophotometry.

The kidney, liver and heart tissues collected from the sacrificed rats were fixed in paraffin and sliced into 4 μm-thick sections. Deparaffined and stained with Hematoxylin-Eosin (H-E) stain, the sections were observed under a light microscope at magnifications of 400× and 1000× (histopathological examination) in the Department of Pathology, Bangabandhu Sheikh Mujib Medical University (BSMMU), Bangladesh.

Statistical analysis

Multiple comparisons of the means of liver function markers in the control group and Goreisan/Wulingsan groups were performed at each dose using a two-sided Dunnett test with SPSS software (version 17.0; SPSS, Inc., Chicago, Illinois, USA). A p-value of less than 0.05 was considered to be statistically significant.

Results

Visual rat inspections and rat survival data

All of the rats in both the control group and Goreisan/Wulingsan groups consumed their medication completely. No abnormal habitation change or feeding activity was observed in any of the rats over the 25-day study period, nor did any of the rats suffer constipation. In addition, no abnormal movement, excitation or lethargy was observed among the rats in the study group. Since all of the rats survived for the 25-day study period with an up to 8 mg/g dose of Goreisan/Wulingsan, we were unable to establish a lethal dose within this range.

Biochemical liver function tests using rat sera

The results of biochemical liver function tests are shown in Fig. 1. The mean and SD of serum AST levels (IU/L) in the control group and the rats fed 1, 2, 4, and 8 mg/g/day of Goreisan/Wulingsan were 43.8 ± 16.0, 34.9 ± 9.5, 45.3 ± 10.3, 46.1 ± 23.2, and 88.4 ± 21.6, respectively. The mean and SD of serum ALT levels (IU/L) in the control group and the rats fed 1, 2, 4, and 8 mg/g/day of Goreisan/Wulingsan were 29.3 ± 8.0, 18.6 ± 3.2, 27.5 ± 8.9, 26.1 ± 5.6, and 48.0 ± 7.5, respectively. No statistically significant elevation of AST or ALT levels was observed between the control group and the various Goreisan/Wulingsan groups, except at the highest dose of 8 mg/g/day (AST: $p = 0.001$, ALT: $p < 0.001$). The mean and SD of serum ALP levels (IU/L) in the control group and the rats fed 1, 2, 4, and 8 mg/g/day of Goreisan/Wulingsan were 279.0 ± 58.9, 268.5 ± 16.5, 220.7 ± 89.5, 284.9 ± 71.5, and 347.8 ± 60.1, respectively. With respect to ALP levels, no statistically significant difference was observed between the control group and any of the Goreisan/Wulingsan groups.

Histopathological examination of rat tissue

The photomicrographs of H-E stained histopathological sections of kidney, liver and heart tissues are shown in Fig. 2. No histopathological change was observed between the control group and the Goreisan/Wulingsan groups in any of the examined tissues at doses of 1 to 8 mg/g/day.

Discussion

This is the first study to verify the safety of Goreisan/Wulingsan using a rat model. The recommended pediatric daily dose (0.25 g/kg/day) of commercially available Goreisan/Wulingsan powder, including excipient (Goreisanryo N17, Kotaro Pharmaceutical Co., Ltd., Osaka, Japan) is equivalent to a dose of 0.13 g/kg/day

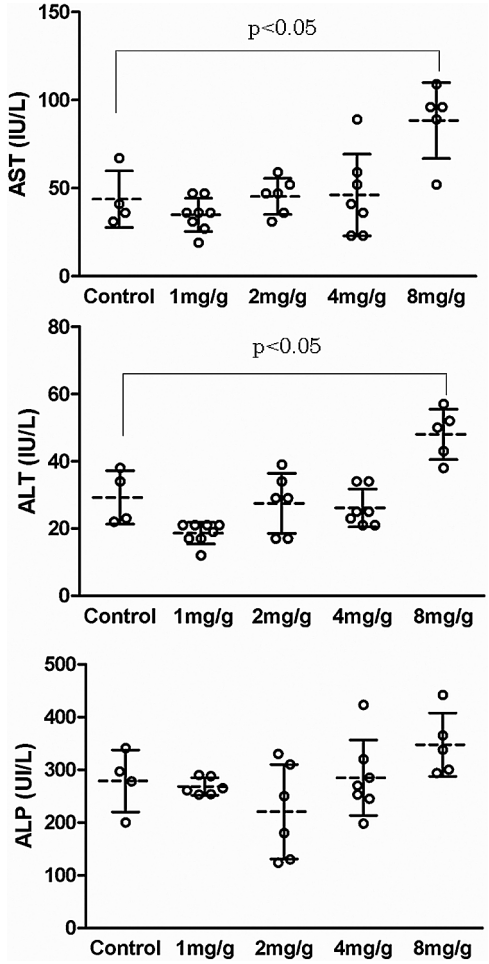

Fig. 1. Plots of biochemical liver function tests.
aspartateaminotransferase (AST); alanine aminotransferase (ALT); and alkaline phosphatase (ALP). These plots represent enzyme levels in the rats of the control group and those of the Goreisan/Wulingsan groups (at doses of 1, 2, 4 and 8 mg/g/day).
The error bars indicate the standard deviations and the dashed lines indicate the mean values for the data.

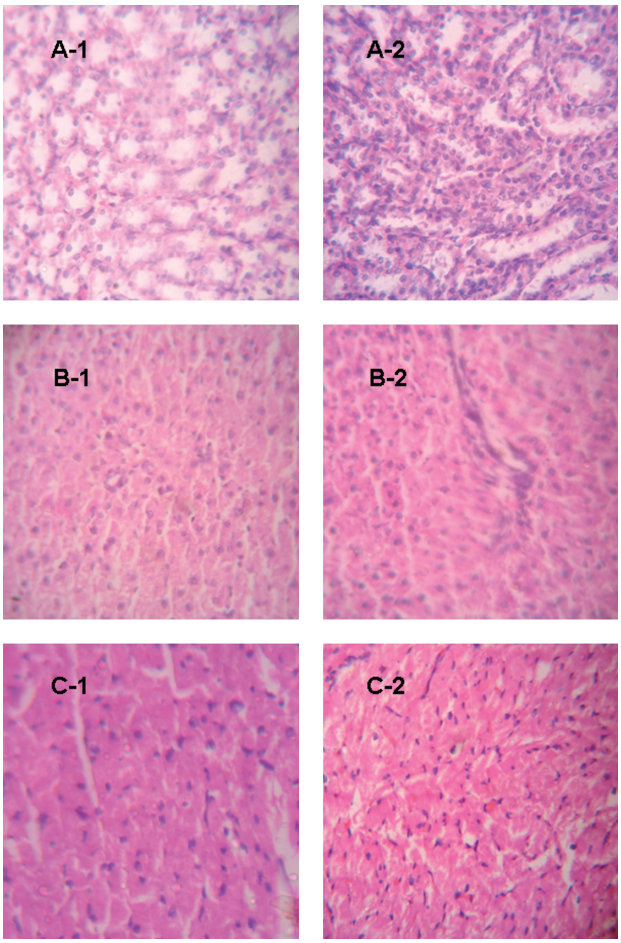

Fig. 2. Histopathological study of kidey, liver and heart tissues collected from rats.
Photomicrographs of Hematoxylin-Eosin (H-E)-stained sections from the organs of rats belonging to the control group and the Goreisan/Wulingsan group at the highest Goreisan/Wulingsan dose of 8 mg/g/day, as observed under a light microscope at 400 fold magnification. A-1: kidney tissue from the control group, A-2: kidney tissue from the Goreisan/Wulingsan group at a dose of 8 mg/g/day. B-1: liver tissue from the control group, B-2: liver tissue from the Goreisan/Wulingsan group at a dose of 8 mg/g/day. C-1: heart tissue from the control group, C-2: heart tissue from the Goreisan/Wulingsan group at a dose of 8 mg/g/day

(0.13 mg/g/day) of dehydrated extracts of the five herbs of Goreisan/Wulingsan (without the excipient) used in this experiment. Therefore, the doses of Goreisan/Wulingsan used for this animal study were 7.7 to 61.5 times higher than the recommended pediatric dose of Goreisan/Wulingsan powder. All of our test rats survived and remained healthy over the 25-day observation period, a result indicating that Goreisan/Wulingsan is also safe for use in various human races worldwide.

All of our test rats consumed the medication and feed pellets completely, and none of them suffered from constipation. Therefore, Goreisan/Wulingsan displayed no major inhibitory effect on intestinal movement, which should be avoided in the treatment of infectious vomiting and diarrhea. Although excitatory and inhibitory effects on the central nervous system of mice have been reported at high doses (more than 0.1 mg/g) of Cinnamaldehyde a chemical component of *Cinnamomumcassia Blume*, one of the herbs in Goreisan/Wulingsan [11], neither excitatory nor inhibitory effects were observed in the central nervous system of our test rats at doses of 1 to 8 mg/g/day. This also suggests the likelihood that clinical doses of Goreisan/Wulingsan cause no adverse effects on the central nervous system in

human subjects.

As for the serum liver function tests, we used human liver function test kits since those for rats were not available in Bangladesh. Therefore, the absolute liver function test values that we obtained cannot be directly compared with liver function test values in rats. However, a comparison of liver function test values among the Goreisan/Wulingsan group with those of the control group might still be useful for evaluating the effects on liver function. With regard to AST and ALT levels, statistically significant elevations of liver enzymes were only observed between the control group and the Goreisan/Wulingsan group at the highest of the drug dose (8 mg/g/day). However, the mean level of liver enzymes at the highest dose was, at most, twice that of the control group, which is a clinically acceptable variance. In addition, no abnormality was detected in the histopathological studies of the liver tissue from rats receiving Goreisan/Wulingsan, even at the highest dose.

Vomiting and diarrhea cause dehydration resulting in decreased renal blood flow, and this could damage kidney tissue and make it more susceptible to renal toxic agents. Hence, renal toxic agents should be avoided in the treatment of vomiting and diarrhea. No pathological change was observed in the kidney tissues of rats fed Goreisan/Wulingsan, at any dosage. Electrolyte imbalance, which is induced by severe vomiting and diarrhea, can lead to occasionally life-threatening arrhythmia, especially in patients with underlying heart disease [12]. Therefore, any medicine which is used to treat vomiting and diarrhea should be tested for heart tissue toxicity. In our histopathological examinations of the rat heart tissue, we observed no adverse change in the Goreisan/Wulingsan groups at any dosage.

Ahn et al. reported a diuretic effect of Goreisan/Wulingsan [7]. If Goreisan/Wulingsan induced a diuretic effect on children with dehydration, it would be very dangerous because of the risk of kidney damage and electrolytic disorders. However, in another study, the urine volume of rats fed Goreisan/Wulingsan was not significantly different from that of control rats (rats fed normal food) for 8 weeks [13]. The diuretic effect of Goreisan/Wulingsan is therefore still controversial. Tei et al. reported that Goreisan/Wulingsan induced a remarkable diuretic effect in mice loaded with excess water, even though it did not induce a diuretic effect in mice with normal water intake. In addition, Goreisan/Wulingsan induced an anti-diuretic effect in mice with dehydration [14]. Tashiro reported a similar modulating effect of Goreisan/Wulingsan on the water balance in a human subject [15]. Goreisan/Wulingsan induced a diuretic effect in a human volunteer loaded with excess water but an anti-diuretic effect in a volunteer suffering from dehydration. Since Goreisan/Wulingsan acts to normalize water balance, it is suitable for the treatment of vomiting and diarrhea in patients with dehydration.

Regarding the limitations of our study, we did not assess renal function or electrolytes using the rat serum. Haranaka et al. reported that the creatinine clearance of rats fed Goreisan/Wulingsan for 1 month was within a normal range [13]. Watabe et al. reported that concentrations of potassium, sodium, calcium, magnesium, and chloride in the sera of rats fed with Goreisan/Wulingsan for 1 month were similar to those in the control group [16]. As for feeding activity, we only made visual observations. Orita et al. reported that the amount of consumed food and the body weight of rats fed Goreisan/Wulingsan for 8 weeks were not significantly different from those in the control group (rats fed normal food) [17].

Our results can be summarized as follows. High doses (7.7 to 61.5 times higher than the recommended pediatric dose) of Goreisan/Wulingsan were given to rats. All the animals survived and remained healthy over the 25-day observation period. In addition, we observed no inhibition of intestinal movement, no neurologically abnormal activity, no marked elevation of liver enzymes, and no pathological tissue change (kidney, liver and heart tissue). These results indicate the safety of this Japanese-Chinese herbal medicine in human patients suffering from vomiting and diarrhea, a finding that may open the door for the use of Goreisan/Wulingsan outside East Asia, especially in developing countries in tropical area of Africa and South Asia where huge numbers of children die from acute gastroenteritis.

On the basis of our results, a clinical trial of Goreisan/Wulingsan in Bangladeshi children with acute vomiting and diarrhea was approved by the Ethical Committee of Institute of Child and Mother Health, Dhaka, Bangladesh. The trial was safely conducted between May 2008 and May 2009 (manuscript under preparation). The efficacy of Goreisan/Wulingsan on acute gastroenteritis (vomiting and diarrhea) is promising from the view point of both our clinical experience and an open label controlled study conducted in Japan [5]. In a mice model, saline purgative-induced diarrhea was reduced by Goreisan/Wulingsan [18]. This finding suggests that Goreisan/Wulingsan modulates the water permeability of the intestinal membrane and is effective in reducing secretory diarrhea. Since Goreisan/Wulingsan is empirically effective for both vomiting and diarrhea, it is a promising candidate for adjunctive therapy using oral rehydration salts (ORS). Vomiting control makes ORS therapy successful, while diarrhea control prevents increases in the level of dehydration. In

addition, the price of Goreisanryo N-17 (Kotaro Pharmaceutical Co., Ltd.) is only 0.2 USD/day per child with an 8 kg body weight. Since Goreisan/Wulingsan is affordable in poor countries, it is expected to be a popular choice for the control of diarrhea in children living in tropical, developing countries.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the results of this study.

Author's Contribution

SA designed the study as a principal investigator, interpreted data, and drafted the manuscript. RU performed statistical analyses, interpreted data and edited the manuscript. MH performed the histopathological study. ALK designed the study and interpreted data. MZR and MSR designed and performed animal experiments. SH supervised and performed statistical analyses. MAR designed the study and supervised the animal experiments.

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