A Study on the Skin Irritation Toxicity Test of Processed Sulfur in New Zealand White Rabbit

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Objectives: This study was performed to evaluate the skin irritation toxicity of processed sulfur.

Methods: All experiments were conducted at Medvill (Korea), an institution authorized to perform non-clinical studies, under the Good Laboratory Practice (GLP) regulations. In order to investigate skin irritation toxicity of processed sulfur, we divided the back of six rabbits into two control sites and two test sites. One of each of the two control and test sites was then designated abraded sites and intact sites. In test sites, 0.5 g of processed sulfur was applied to the back of the rabbit for 24 hours, and in control sites, 0.5 g of sterile distilled water was applied in the same way. We observed and evaluated mortality, weight, general symptoms, and skin irritation toxicity. This study was conducted with the approval of the Animal Ethics Committee (Approval number: IAC2020-1549).

Results: In all experiments, no dead animals were observed. In all cases, skin coloration was observed at 24 hours after processed sulfur administration. This coloration lasted up to 48 hours and is believed to be the effect of the administration of test substances. Weight measurement indicated that weight was lost 72 hours after administration in three cases, but this is considered an accidental weight change. Normal weight gain was observed in the remaining subjects. In all animals, no skin irritation toxicity was observed, and the primary irritation index (P.I.I) was calculated as 0.0 according to Draize’s evaluation method.

Conclusion: The above findings suggest that it is relatively safe to apply a processed sulfur to the skin. Further research on this topic is needed to provide more specific evidence.

Keywords: herbal medicine, skin irritation toxicity, processed sulfur, toxicity test

INTRODUCTION

In the research and development of oriental medicine formulations, intra-laboratory verification of their effects is an important way to establish evidence-based oriental medicine [1].

Despite frequent use of medicinal plants, few scientific studies have been conducted to determine the safety of traditional medicinal materials. To determine the safety of medicines and plant products for human consumption, systematic toxicological studies should be conducted to predict toxicity and establish safety criteria in the human body [2]. Drug processing is a traditional pharmaceutical technology in China and plays an important role in reducing the toxicity of traditional drugs. Through long practice, drug processing technology has been developed to eliminate toxicity and side effects of drugs [3].
Sulfur is the third most abundant mineral in our body and is essential for life. Sulfur is a component of the essential amino acids used to make proteins for cells, tissues, hormones, enzymes, and antibodies. Sulfur is one of the mineral drugs traditionally used in herbal medicine. However, direct administration of sulfur to the human body generally causes strong toxic side effects. Accordingly, after processing sulfur to remove the poison, the remaining product, processed sulfur, has been developed for medicinal purposes.

This study was performed to examine the safety of processed sulfur through skin irritation toxicity testing. The testing methods included observation of mortality, body weight changes, general symptoms, and evaluation of the administration site in 12-week-old New Zealand White (NZW) Rabbits.

The current research trend on skin irritation toxicity testing of extracts is to study acute and subacute toxicity through Good Laboratory Practice (GLP) regulations. All experiments for this study were conducted by Medvill (Korea), an institution authorized to perform non-clinical studies, under the GLP regulations.

### MATERIALS AND METHODS

The processed sulfur was prepared in the sterilization room of Wonkwang University Gwangju Korean medical hospital. The processed sulfur was finely ground using a mortar. After using an electronic scale and mixing it with corn oil, it was prepared with normal potency.

The animals used in this study were 12-week-old NZW Rabbits. NZW Rabbits are widely used in safety tests in the field of medicine, so the results can be easily compared with many other databases. The weight range of the rabbits was 2,180.3 g-2,534.3 g and 2,144.1 g-2,621.6 g, when introduced and administered. All animals were observed once a day for 72 hours after administration of the processed sulfur, and the weight of each individual was measured at the end of the observation. In addition, at 24 hours and 72 hours after administration of the processed sulfur, the degree of erythema, eschar, and edema formation was observed and evaluated according to Table 1, and the primary irritation index (P.I.I) was calculated. Skin irritation toxicity was determined according to Table 2 [4, 5].

The laboratory temperature was 20.0°C ± 3.0°C and the humidity was set to 50.0% ± 20.0%. Ventilation was performed 10-20 times per hour, the lighting cycle was adjusted every 12 hours, and the illuminance was set to 150-300 Lux. The cages were stainless steel, measuring (450 W × 405 D × 600 H) mm, and housed one rabbit per cage. The temperature and humidity of the laboratory were measured every 30 minutes by an auto-

### Table 1. Evaluation of skin reaction

| (1) Erythema and eschar formation | Rating   |
|-----------------------------------|----------|
| No erythema at all                | 0        |
| Very light erythema that can be barely seen with the naked eye. | 1        |
| Definitely erythema.             | 2        |
| Kind of severe erythema.         | 3        |
| Severe erythema and light eschar. | 4        |
| Total possible erythema score.   | 4        |

| (2) Edema formation | Rating   |
|---------------------|----------|
| No edema at all     | 0        |
| Very light edema that can be barely seen with the naked eye. | 1        |
| Light edema that distinguishes the marginal area due to swelling. | 2        |
| Normal edema (About 1 mm of swollen edema.) | 3      |
| Severe edema (It swells up more than 1 mm and extends out of the exposed area.) | 4      |
| Total possible edema score. | 4        |

### Table 2. Rating table of skin irritation toxicity

| Primary irritation index (P.I.I) | Rating  |
|---------------------------------|---------|
| 0.0-0.5                         | Non stimulation |
| 0.6-2.0                         | Light stimulation |
| 2.1-5.0                         | Moderate stimulation |
| 5.1-8.0                         | Strong stimulation |

### Table 3. Animal grouping

| Group | Sex | Serial number | Number of animal | Dose | Area of administration | Route of administration |
|-------|-----|---------------|-----------------|------|------------------------|-------------------------|
| G1    | Male | 1101-1106     | 6               | Test substance: 0.5 g | Test site: 2 | Skin         |
|       |      |               |                 | Control substance: 0.5 mL | Control site: 2 |             |

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matic measuring instrument, and other environmental conditions were measured according to the standard guidelines. As a result of environmental measurement, there was no change that was considered to affect the test.

Radiation sterilized feed for experimental animals was consumed, and reverse osmotic system filtered water was provided. No indications were found that feed and water influenced the test. Groupings were done after five days of acclimatization. The back area was shaved to select animals with clean and healthy skin, and group separation was performed using a random method so that the average weight and standard deviation between groups were uniform (Table 3).

24 hours before administration, the dorsal area was shaved [approximately (15 × 15) cm$^2$] and before administration [(2.5 × 2.5) cm$^2$] was divided and marked into two test sites and two control sites. For each individual, the test sites and control sites were divided into abraded and non-abraded. Only the epidermis was damaged, leaving the dermis undamaged, and bloodless abrasions were applied (Fig. 1). Then, after applying 0.5 g of the processed sulfur to gauze, it was attached to the test sites so that it contacted both the abraded and non-abraded locations. In the lower control sites, 0.5 mL of sterile distilled water was applied to gauze and then attached in the same way. The administration site was fixed using a non-stimulating tape (Tegaderm, 3M, Korea) and an elastic bandage (Coban, 3M, Korea). The gauze was removed 24 hours later and the processed sulfur remaining on the skin was cleaned using sterilized distilled water. This study was conducted under the approval of the Institutional Animal Ethics Committee of Medvill Co., Ltd. (Korea)

RESULTS

In this study, no animals died during the experiment (Table 4). Skin coloration was observed in all cases for 24 hours after administration and up to 48 hours. In addition, weight loss was observed when measured at 72 hours after administration in three cases, and normal weight gain was observed in all other animals (Table 5). Finally, no skin reaction was observed in any

| Table 4. Mortality and clinical sign |
|-------------------|-----------------|------------------|
| Animal number | Day(s) after application | 0 | 1 | 2 | 3 | Mortality |
| 1101 | N | K40 (09.01.08) | K40 (09.01.08) | N | 0% (0/6)$^*$ |
| 1102 | N | K40 (09.01.08) | K40 (09.01.08) | N |
| 1103 | N | K40 (09.01.08) | K40 (09.01.08) | N |
| 1104 | N | K40 (09.01.08) | K40 (09.01.08) | N |
| 1105 | N | K40 (09.01.08) | K40 (09.01.08) | N |
| 1106 | N | K40 (09.01.08) | K40 (09.01.08) | N |

N, normal; $^*$Number of dead animals/Number of total animals.
K40 (09.01.08) : Coloring of skin (Black.Slight.Black).

| Table 5. Body weight |
|-------------------|-----------------|------------------|
| Animal number | Hour(s) after application | Weight gains |
| 1101 | 0 | 2,621.6 | 2,699.3 | 77.7 |
| 1102 | 72 | 2,580.2 | 2,425.4 | -154.8 (6.0%) |
| 1103 | 2,589.1 | 2,583.9 | -5.2 (0.2%) |
| 1104 | 2,500.1 | 2,426.6 | -73.5 (2.9%) |
| 1105 | 2,144.1 | 2,186.8 | 42.7 |
| 1106 | 2,437.1 | 2,587.5 | 150.4 |
| Mean | 2,478.7 | 2,484.9 | 6.2 |
| S.D. | 177.2 | 180.1 | 109.2 |

S.D, standard deviation.
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animal at the administration site 24 hours and 72 hours after administration of the processed sulfur (Figs. 2, 3) (Table 6).

**DISCUSSION**

Sulfur is a bright yellow crystalline solid or powder. It is the seventh most abundant mineral in our body. A person weighing about 70 kg contains about 140 g of sulfur. It is obtained mainly through the form of sulfur-containing amino acids (SAA) such as methionine, cysteine, and taurine, as well as the intake of glucosinolates found in cruciferous vegetables such as cabbage and cauliflower [6].

![Figure 2. Skin photograph at 24 hours after application of test substance.](image1)

![Figure 3. Skin photograph at 72 hours after application of test substance.](image2)

**Table 6. Evaluation of skin irritation toxicity**

| Site        | Skin reaction          | Observation time | Individual scores – Animal number | Total |
|-------------|------------------------|------------------|-----------------------------------|-------|
|             |                        |                  | 1101 1102 1103 1104 1105 1106     |       |
| Control site| Erythema/Eschar formation | Intact 24 H     | 0 0 0 0 0 0                       | 0     |
|             |                        | 72 H             | 0 0 0 0 0 0                       | 0     |
|             |                        | Abraded 24 H     | 0 0 0 0 0 0                       | 0     |
|             |                        | 72 H             | 0 0 0 0 0 0                       | 0     |
|             | Edema formation        | Intact 24 H      | 0 0 0 0 0 0                       | 0     |
|             |                        | 72 H             | 0 0 0 0 0 0                       | 0     |
|             |                        | Abraded 24 H     | 0 0 0 0 0 0                       | 0     |
|             |                        | 72 H             | 0 0 0 0 0 0                       | 0     |
| Test site   | Erythema/Eschar formation | Intact 24 H     | 0 0 0 0 0 0                       | 0     |
|             |                        | 72 H             | 0 0 0 0 0 0                       | 0     |
|             |                        | Abraded 24 H     | 0 0 0 0 0 0                       | 0     |
|             |                        | 72 H             | 0 0 0 0 0 0                       | 0     |
|             | Edema formation        | Intact 24 H      | 0 0 0 0 0 0                       | 0     |
|             |                        | 72 H             | 0 0 0 0 0 0                       | 0     |
|             |                        | Abraded 24 H     | 0 0 0 0 0 0                       | 0     |
|             |                        | 72 H             | 0 0 0 0 0 0                       | 0     |
|             | Sum of mean            |                  | 0.0                               |       |
|             | P.I.I (S**/4***)       |                  | 0.0                               |       |
|             | Test site              | Erythema/Eschar formation | Intact 24 H | 0 0 0 0 0 0 | 0     |
|             |                        | 72 H             | 0 0 0 0 0 0                       | 0     |
|             |                        | Abraded 24 H     | 0 0 0 0 0 0                       | 0     |
|             |                        | 72 H             | 0 0 0 0 0 0                       | 0     |
|             | Edema formation        | Intact 24 H      | 0 0 0 0 0 0                       | 0     |
|             |                        | 72 H             | 0 0 0 0 0 0                       | 0     |
|             |                        | Abraded 24 H     | 0 0 0 0 0 0                       | 0     |
|             |                        | 72 H             | 0 0 0 0 0 0                       | 0     |
|             | Sum of mean*           |                  | 0.0                               |       |
|             | P.I.I (S**/4***)       |                  | 0.0                               |       |

H, hours; P.I.I, Primary irritation index; *24 and 72 h readings; **Sum of mean; ***[(Number of test sits = 12) × (Scoring intervals = 2)] / 6.

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In Dongui Bogam, sulfur is mainly used for the treatment of abdominal masses, pathogenic Qi in the pit of the stomach, stagnation of cold Qi, chronic cold syndrome in the back and the kidneys, loss of sensation due to cold wind, and coldness, pain, and loss of power in the legs. It fortifies the sinews and bones, tonifies yang Qi, removes balding, malignant furuncles, infantile malnutrition affecting the nose and other conditions in the external genitals, and kills scabies parasites [7].

Sulfur is widely used to detoxify the body, treat scabs, heal wounds, and remove itching sensations [8-10].

Recent studies have reported that sulfo compounds inhibit cancer cell growth. Kong et al. [11] suggested that in humans, the treatment of gastric cancer cells (AGS) with extracts from young radishes with high organosulfur glucosinolate content inhibits cancer cell growth. In addition, Bak et al. [12] reported that treating HT-29 human colon cancer cells with sulfur-treated radish kimchi extract also inhibits cancer cell growth. Choi and Kim [13] reported that, when diverse cancer cells were treated with extracts from a hot water extraction from regular ducks or organic-sulfur-fed ducks, a noticeable effect in proliferation inhibition was seen in the cells treated with the extract from organic-sulfur-fed ducks.

In previous studies, inorganic sulfur has been proven to inhibit the proliferation of breast cancer cells. Inorganic sulfur was found to reduce cell proliferation by inhibiting the expression and activation of epidermal growth factor receptor (EGFR) and increasing the expression of Bcl-2-associated X (Bax) in estrogen-independent breast cancer (MDA-MB-231) [14]. Another study investigated the inhibitory effect of sulfur on prostate cancer (PCa) in vivo. In that research, prostate tumors were developed by injecting 22 Rv1 or DU 145 PCa cells into sulfur treated and untreated nude mice. The results showed that sulfur inhibits the growth of androgen-independent prostate cancer in vivo [15]. In addition, it has been reported that the intake of refined inorganic sulfur reduces the side effects of radiation therapy in cancer patients [16]. However, direct administration of sulfur to the human body generally causes toxic side effects. Therefore, processed sulfur has been developed for medicinal purposes.

Toxicity testing provides important data in evaluating the stability of the drug [17]. Although processed sulfurs have been used in hospitals, safety studies on processed sulfurs are insufficient. In a previous study, Kim et al. reported that administration of 500-2,000 mg/kg of super key (processed sulfur) did not cause any changes in the weights of Sprague-Dawley rats. It also did not affect mortality, indicating that the lethal dose of the super key was more than 2,000 mg/kg [18]. This study was conducted to provide objective safety data for skin irritation toxicity of processed sulfur. 0.5 g of processed sulfur was administered to the back of the experimental group, and 0.5 g of normal saline was administered to the back of the control group. No deaths occurred in any groups, and skin coloration and weight loss were observed in the experimental group. The weight change was considered accidental rather than a result of the administration of processed sulfur, considering the general symptoms and small degree of loss (0.2-6.0%).

Animal testing is the most fundamental method of evaluating the safety of substances to be used for medical purposes [19]. For that reason, the Korea Food & Drug Administration of Korea has testing protocol guidelines for the study of toxicity, and all experiments should be conducted following GLP regulations [20]. The results of this study following this protocol and regulation showed that no skin irritation toxicity was observed after administration of the processed sulfur. In addition, no deaths or significant weight changes occurred, indicating that processed sulfur is safe as a non-irrigational substance. However, this study is limited in that the number of experimental populations is relatively small and the chronic effects of continuous skin stimulation cannot be observed. Therefore, more hematology and blood chemistry research are needed for accurate evaluation of processed sulfur’s skin irritation toxicity in the future.

**CONCLUSION**

The results of this study show that processed sulfur’s skin irritation toxicity does not affect mortality, weight, and primary irritation index. Although coloration occurs for 24-48 hours after processed sulfur administration, it does not cause other symptoms. From the results of this experiment, the processed sulfur is considered a “non-irritating” material.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

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