Intramuscular Single-dose Toxicity Test of *Bufonis venonum* Pharmacopuncture in Sprague-Dawley Rats

Kwang-Ho Lee, Seung-Ho Sun, Jun-Sang Yu, Ki-Rok Kwon

**Abstract**

**Objectives:** *Bufonis venonum* (BV) is the dried white secretions of the auricular and skin glands of the toads Bufo bufo gargarizans or Bufo melanosticus Schneider. This study was performed to evaluate the toxicity of intramuscularly-administered *Bufonis venonum* pharmacopuncture (BVP) and to calculate its approximate lethality through a single-dose test with Sprague-Dawley (SD) rats.

**Methods:** Twenty male and 20 female 6-week-old SD rats were injected intramuscularly with BVP or normal saline. The animals were divided into four groups with five female and five male rats per group: the control group injected with normal saline at 0.5 mL/animal, the low-dosage group injected with 0.125 mL/animal of BVP, the medium-dosage group injected with 0.25 mL/animal of BVP and the high-dosage group injected with 0.5 mL/animal of BVP. All injections were in the left thighs of the rats. After administration, we conducted clinical observations everyday and body weight measurements on days 3, 7 and 14 after the injection. We also carried out hematology, serum biochemistry, and histological observations on day 15 after treatment.

**Results:** No mortalities were observed in any experimental group. No significant changes in weight, hematology, serum biochemistry, and histological observations that could be attributed to the intramuscular injection of BVP were observed in any experimental group.

**Conclusion:** Lethal dose of BVP administered via intramuscular injection in SD rats is over 0.5 mL/animal.

**1. Introduction**

*Bufonis venonum* (BV), called Chan-Su in Chinese or Sum-So in Korean, is toad venom; in particular, it is the dried white secretions of the auricular and skin glands of the toads Bufo bufo gargarizans or Bufo melanosticus Schneider [1-3]. BV has detoxification, anti-inflammatory, cardiotonic, and pain-relief effects [1, 4], and studies on BV have reported local anesthetic actions and anti-cancer effects [5-8].

*Bufonis venonum* pharmacopuncture (BVP) is a pharmacopuncture that is produced by using various substances extracted from the toad venom. Choi et al. [9] reported recently that BVP had therapeutic potential for treating neuropsychiatric disorders such as anxiety or depression disorder, but no side effects or toxicity of BVP have been reported so far. For that reason, we conducted an intramuscular single-dose toxicity test of BVP in Sprague-Dawley (SD) rats to determine the safety of its use safe and to estimating its appropriate dosage.

**2. Materials and Methods**
Twenty-four SD rats of each gender were obtained from Orientbio Inc. (Gyeong-gi, Korea) at 5 weeks of age and were used after a week of quarantine and acclimatization. The animals were housed in a room maintained at 21.1 — 24.1°C under a relative humidity of 40.7% — 64.5%. The room was illuminated with artificial lighting from 07:00 to 19:00 hours and had 10 — 15 air changes per hour. Three animals per cage were housed in suspended stainless-steel wire-mesh cages and were allowed sterilized tap water and commercial rodent chow (Teklad Certified Irradiated Global 18% Protein Rodent Diet 2918C, Harlan Laboratories, Inc., U.S.A.). The variance in the numerical data was checked by using the Bartlett test. If the variance was homogeneous, the data were subjected to a one-way analysis of variance (ANOVA). If either of the tests showed a significant difference among the groups, the data were analyzed using the multiple comparison procedure of the Dunnett test. If not, they were analyzed using the Kruskal-Wallis non-parametric ANOVA test ($P<0.05$).

### 3. Results

No treatment-related mortalities, clinical signs or weight changes occurred in either the control animals or the animals treated with any dose of BVP during the observation period (Tables 1, 2). On the hematological examination (Table 3), one female in the medium-dosage group showed a significant change; however, the change was not dose-dependent; the change seemed to have occurred sporadically. The blood chemistry tests (Table 4) showed no significant changes.

The necropsy examinations (Tables 5, 6) showed no abnormalities. Moreover, on the histopathological examination, one male in the control group and one female in the low-dosage group showed abnormal changes, but those changes were not dose-dependent. Thus, they were deemed not be important toxicological changes.

### 4. Discussion

BV has some toxic ingredients that can induce serious effects, including bradycardia, atrioventricular conduction block, ventricular tachycardia, ventricular fibrillation, and sudden death [10]. Bufadienolides, such as bufalin, cinobufagin and resibufogenin, which are major sources of BV, are known to increase vasoconstriction, vascular resistance and blood pressure probably by inhibiting Na, K-adenosine triphosphate (ATP) ase activity [11, 12], and these substances have recently been reported to have a strong surface anesthetic activity, cytotoxic effect and differentiation-apoptosis activity on murine leukemia human acute promyelocytic leukemia (HL-60) cells [13]. In addition, BV includes bufotenine, an indole alkaloid that produces effects such as aphrodisia and hallucination, and serotonin, which is involved in various psychiatric disorders such as depression, anxiety, chronic obsession syndrome and impulsivity [14-16]. Thus, appropriate dosage and careful use are very important for the safe use of BV [2].

For the above reasons, we conducted an intramuscular...
Table 1 Summary of mortalities

| Sex   | Group / Dose (mL/animal) | No. of animals | Days after dosing | Mortality |
|-------|--------------------------|----------------|-------------------|-----------|
|       |                          | 0  | 1  | 2  | 3  | 4  | 5  | 6  | 7  | 8  | 9  | 10 | 11 | 12 | 13 | 14 |
| Male  | G1(0)                    | 5  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0/5 |
|       | G2 (0.125)               | 5  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0/5 |
|       | G3 (0.25)                | 5  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0/5 |
|       | G4 (0.5)                 | 5  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0/5 |
| Female| G1 (0)                   | 5  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0/5 |
|       | G2 (0.125)               | 5  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0/5 |
|       | G3 (0.25)                | 5  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0/5 |
|       | G4 (0.5)                 | 5  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0  | 0/5 |

Table 2 Mean body weights

| Sex   | Group / Dose (mL/animal) | Mean | S.D. | N  | Days after dosing | Gain (g) |
|-------|--------------------------|------|------|----|-------------------|----------|
|       |                          |      |      |    | 0  | 3  | 7  | 14 | 0 — 14 |
| Male  | G1 (0)                   | Mean 191.5 | 4.8 | 5  | 219.1 | 10.3 | 315.4 | 123.9 |
|       |                          | S.D. 6.7 | 5  | 255.3 | 17.9 | 5  | 13.6 |
|       |                          | N 5 | 5  | 5  | 5  | 5 |
|       |                          | Mean 190.0 | 5.5 | 5  | 217.9 | 9.5 | 314.7 | 124.7 |
|       |                          | S.D. 5.4 | 5  | 256.1 | 15.3 | 5  | 10.6 |
|       |                          | N 5 | 5  | 5  | 5  | 5 |
|       |                          | Mean 188.8 | 3.0 | 5  | 214.0 | 6.3 | 309.6 | 120.8 |
|       |                          | S.D. 4.3 | 5  | 250.2 | 9.0 | 5  | 7.1 |
|       |                          | N 5 | 5  | 5  | 5  | 5 |
|       |                          | Mean 189.9 | 6.7 | 5  | 216.0 | 9.1 | 318.5 | 128.5 |
|       |                          | S.D. 9.1 | 5  | 254.7 | 11.5 | 5  | 11.2 |
|       |                          | N 5 | 5  | 5  | 5  | 5 |
| Female| G1 (0)                   | Mean 157.3 | 8.3 | 5  | 173.0 | 15.5 | 220.5 | 63.2 |
|       |                          | S.D. 11.7 | 5  | 192.0 | 18.8 | 5  | 12.0 |
|       |                          | N 5 | 5  | 5  | 5  | 5 |
|       |                          | Mean 159.1 | 1.8 | 5  | 175.3 | 7.5 | 218.0 | 58.9 |
|       |                          | S.D. 3.2 | 5  | 192.4 | 9.6 | 5  | 11.2 |
|       |                          | N 5 | 5  | 5  | 5  | 5 |
|       |                          | Mean 157.0 | 8.5 | 5  | 173.3 | 8.5 | 220.6 | 63.6 |
|       |                          | S.D. 7.8 | 5  | 192.7 | 12.5 | 5  | 4.5 |
|       |                          | N 5 | 5  | 5  | 5  | 5 |
|       |                          | Mean 157.0 | 7.4 | 5  | 172.1 | 10.9 | 212.8 | 55.8 |
|       |                          | S.D. 8.1 | 5  | 187.1 | 14.9 | 5  | 9.2 |
|       |                          | N 5 | 5  | 5  | 5  | 5 |

S.D., standard deviation; N, number of animals.
### Table 3 Mean hematology parameters

(Male)

| Group/Dose  | Mean RBC (× 10⁶ cells/µL) | Mean HGB (g/dL) | Mean HCT (%) | Mean RBC Indices | Mean PLT (× 10³ cells/µL) | Mean Reti (%) |
|-------------|-----------------------------|-----------------|-------------|------------------|--------------------------|-------------|
| G1 (0)      | 7.23                        | 14.8            | 42.4        | 58.6             | 20.5                     | 34.9        |
|             | 0.10                        | 0.4             | 1.2         | 1.9              | 0.7                      | 0.9         |
|             | 5                           | 5               | 5           | 5                | 5                        | 5           |

| G2 (0.125) | 7.56                        | 15.3            | 44.2        | 58.4             | 20.2                     | 34.5        |
|            | 0.25                        | 0.4             | 1.7         | 1.2              | 0.4                      | 0.5         |
|            | 5                           | 5               | 5           | 5                | 5                        | 5           |

| G3 (0.25)  | 7.32                        | 14.8            | 42.5        | 58.0             | 20.2                     | 34.8        |
|            | 0.30                        | 0.6             | 1.7         | 0.8              | 0.4                      | 0.6         |
|            | 5                           | 5               | 5           | 5                | 5                        | 5           |

| G4 (0.5)   | 7.34                        | 14.9            | 43.4        | 59.2             | 20.3                     | 34.3        |
|            | 0.27                        | 0.5             | 1.6         | 1.7              | 0.7                      | 0.4         |
|            | 5                           | 5               | 5           | 5                | 5                        | 5           |

| Group/Dose  | Mean WBC (× 10³ cells/µL) | Mean WBC Differential Counting (%) | Mean PT (sec) | Mean APTT (sec) |
|-------------|---------------------------|-----------------------------------|---------------|-----------------|
| G1 (0)      | 8.29                      | 18.7                              | 1.4           | 0.1             |
|             | 2.35                      | 4.2                               | 0.3           | 0.2             |
|             | 5                         | 5                                 | 5             | 5               |

| G2 (0.125) | 7.71                      | 14.7                              | 1.8           | 0.5             |
|            | 1.28                      | 1.9                               | 0.5           | 0.1             |
|            | 5                         | 5                                 | 5             | 5               |

| G3 (0.25)  | 7.62                      | 17.4                              | 1.6           | 0.4             |
|            | 2.28                      | 6.9                               | 0.4           | 0.1             |
|            | 5                         | 5                                 | 5             | 5               |

| G4 (0.5)   | 9.32                      | 16.2                              | 1.6           | 0.5             |
|            | 2.24                      | 3.1                               | 0.4           | 0.1             |
|            | 5                         | 5                                 | 5             | 5               |

(Female)

| Group/Dose  | Mean RBC (× 10⁶ cells/µL) | Mean HGB (g/dL) | Mean HCT (%) | Mean RBC Indices | Mean PLT (× 10³ cells/µL) | Mean Reti (%) |
|-------------|---------------------------|-----------------|-------------|------------------|--------------------------|-------------|
| G1 (0)      | 7.72                      | 15.5            | 42.8        | 55.4             | 20.0                     | 36.1        |
|             | 0.16                      | 0.5             | 1.5         | 1.5              | 0.4                      | 0.2         |
|             | 5                         | 5               | 5           | 5                | 5                        | 5           |

| G2 (0.125) | 7.63                      | 15.4            | 42.9        | 56.3             | 20.2                     | 35.9        |
|            | 0.25                      | 0.4             | 1.4         | 1.9              | 0.6                      | 0.5         |
|            | 5                         | 5               | 5           | 5                | 5                        | 5           |

(Continued)
| Group/ Dose (mL/animal) | Mean S.D. N | WBC (x 10^3 cells/μL) | WBC Differential Counting (%) | PT (sec) | APTT (sec) |
|------------------------|-------------|------------------------|-------------------------------|---------|-----------|
|                        |             |                        | NEU | LYM | MONO | EOS | BASO |                |         |
| (0)                    |             |                        | 5.24 | 15.3 | 80.9 | 1.6 | 1.6 | 0.1 | 18.4 | 14.8 |
| G1                     |             |                        | 1.25 | 5.7 | 6.8 | 0.8 | 0.4 | 0.1 | 0.3 | 1.4 |
| N                      |             |                        | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| (0.125)                |             |                        | 4.83 | 10.7 | 85.8 | 1.5 | 1.0 | 0.2 | 17.8 | 13.4 |
| G2                     |             |                        | 1.05 | 1.7 | 1.2 | 0.5 | 0.2 | 0.1 | 0.3 | 1.8 |
| N                      |             |                        | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| (0.25)                 |             |                        | 3.63 | 6.0 | 6.5 | 0.4 | 0.5 | 0.1 | 1.2 | 0.6 |
| G3                     |             |                        | 3.63 | 6.0 | 6.5 | 0.4 | 0.5 | 0.1 | 1.2 | 0.6 |
| N                      |             |                        | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| (0.5)                  |             |                        | 2.40 | 1.9 | 2.2 | 0.3 | 0.3 | 0.0 | 0.9 | 1.5 |
| G4                     |             |                        | 2.40 | 1.9 | 2.2 | 0.3 | 0.3 | 0.0 | 0.9 | 1.5 |
| N                      |             |                        | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |

Significantly different from control by Dunnett’s t-test: *P < 0.05.

S.D., standard deviation; N, number of animals; RBC, red cell; HGB, hemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; PLT, platelet; Reti, reticulocytes; WBC, white cell; NEU, neutrophils; LYM, lymphocytes; MONO, monocytes; EOS, eosinophils; BASO, basophils; PT, prothrombin time; APTT, activated partial thromboplastin time.

Table 4 Mean clinical chemistry

(Male)

| Group/ Dose (mL/animal) | Mean S.D. N | ALT (U/L) | AST (U/L) | ALP (U/L) | GGT (U/L) | Glu (mg/dL) | BUN (mg/dL) | Crea (mg/dL) | T-Bili (mg/dL) | T-Chol (mg/dL) |
|------------------------|-------------|-----------|-----------|-----------|-----------|-------------|-------------|-------------|----------------|----------------|
| (0)                    |             | 30.0      | 92.1      | 937.3     | 0.41      | 113         | 12.2        | 0.41        | 0.03           | 70             |
|                        |             | 2.2       | 12.2      | 176.7     | 0.08      | 15          | 1.1         | 0.01        | 0.03           | 6              |
|                        |             | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| (0.125)                |             | 32.1      | 107.3     | 851.6     | 0.39      | 116         | 11.8        | 0.39        | 0.02           | 66             |
|                        |             | 4.2       | 22.0      | 174.7     | 0.05      | 12          | 1.0         | 0.02        | 0.01           | 15             |
|                        |             | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| (0.25)                 |             | 32.3      | 105.6     | 917.2     | 0.41      | 109         | 12.8        | 0.40        | 0.01           | 68             |
|                        |             | 4.5       | 22.3      | 117.0     | 0.14      | 18          | 2.3         | 0.04        | 0.01           | 19             |
|                        |             | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| (0.5)                  |             | 28.5      | 118.6     | 777.7     | 0.45      | 117         | 12.2        | 0.40        | 0.02           | 69             |
|                        |             | 3.2       | 15.3      | 169.7     | 0.12      | 8           | 0.8         | 0.02        | 0.01           | 18             |
|                        |             | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |

(Continued)
| Group/ Dose (mL/animal) | Mean | S.D. | N  |
|-------------------------|------|------|----|
|                         | TG (mg/dL) | TP (g/dL) | Alb (g/dL) | A/G ratio | P (mg/dL) | Ca (mg/dL) | Na (mmol/L) | K (mmol/L) | Cl (mmol/L) |
| G1 (0)                  | Mean 37 | 5.4 | 2.3 | 0.76 | 8.70 | 9.7 | 140 | 4.8 | 105 |
|                         | S.D. 16 | 0.2 | 0.1 | 0.03 | 0.66 | 0.3 | 0 | 0.6 | 2 |
|                         | N 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| G2 (0.125)              | Mean 40 | 5.4 | 2.3 | 0.76 | 8.69 | 10.0 | 141 | 4.7 | 105 |
|                         | S.D. 24 | 0.3 | 0.1 | 0.05 | 0.57 | 0.4 | 1 | 0.3 | 1 |
|                         | N 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| G3 (0.25)               | Mean 33 | 5.3 | 2.3 | 0.76 | 8.55 | 9.9 | 140 | 4.6 | 105 |
|                         | S.D. 16 | 0.0 | 0.1 | 0.04 | 0.38 | 0.2 | 1 | 0.3 | 1 |
|                         | N 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| G4 (0.5)                | Mean 37 | 5.2 | 2.3 | 0.80 | 8.65 | 9.7 | 140 | 4.9 | 105 |
|                         | S.D. 13 | 0.1 | 0.1 | 0.09 | 0.28 | 0.2 | 1 | 0.2 | 1 |
|                         | N 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |

(Female)

| Group/ Dose (mL/animal) | Mean | S.D. | N  |
|-------------------------|------|------|----|
|                         | ALT (U/L) | AST (U/L) | ALP (U/L) | GGT (U/L) | Glu (mg/dL) | BUN (mg/dL) | Crea (mg/dL) | T-Bili (mg/dL) | T-Chol (mg/dL) |
| G1 (0)                  | Mean 26.8 | 90.3 | 572.4 | 0.65 | 116 | 13.2 | 0.42 | 0.01 | 77 |
|                         | S.D. 1.6 | 10.1 | 196.5 | 0.21 | 3 | 1.0 | 0.02 | 0.01 | 13 |
|                         | N 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| G2 (0.125)              | Mean 24.1 | 90.3 | 456.9 | 0.52 | 117 | 13.7 | 0.41 | 0.01 | 76 |
|                         | S.D. 3.7 | 20.5 | 90.9 | 0.22 | 7 | 1.5 | 0.03 | 0.01 | 10 |
|                         | N 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| G3 (0.25)               | Mean 22.1 | 94.8 | 531.7 | 0.60 | 118 | 11.8 | 0.43 | 0.01 | 89 |
|                         | S.D. 3.8 | 27.1 | 101.4 | 0.11 | 4 | 1.3 | 0.02 | 0.00 | 15 |
|                         | N 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| G4 (0.5)                | Mean 24.6 | 93.3 | 543.8 | 0.52 | 118 | 14.7 | 0.45 | 0.01 | 92 |
|                         | S.D. 3.1 | 12.1 | 150.3 | 0.17 | 5 | 3.2 | 0.04 | 0.01 | 8 |
|                         | N 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |

(Continued)
Table 5 Summary of necropsy findings

|                              | Male |    |    |    |    |    |    |    |    |     |
|------------------------------|------|----|----|----|----|----|----|----|----|------|
| Group                        | G1   | G2 | G3 | G4 | G1 | G2 | G3 | G4 |    |      |
| Dose (mL/animal)             |      |    |    |    |    |    |    |    |    |      |
|                              | 0    | 0.125 | 0.25 | 0.5 | 0 | 0.125 | 0.25 | 0.5 |    |      |
| No. of animals               | 5    | 5  | 5  | 5  | 5 | 5  | 5  | 5  | 5  | 5    |
| Unremarkable findings        | 5    | 5  | 5  | 5  | 5 | 5  | 5  | 5  | 5  | 5    |
| No. of rats examined         | 5    | 5  | 5  | 5  | 5 | 5  | 5  | 5  | 5  | 5    |

External surface and all organs in the body cavity were unremarkable.

Table 6 Summary of histopathological findings

| Organ / Findings | Male |    |    |    |    |    |    |    |    |     |
|------------------|------|----|----|----|----|----|----|----|----|------|
| Group            | G1   | G2 | G3 | G4 | G1 | G2 | G3 | G4 |    |      |
| Dose (mL/animal) | 0    | 0.125 | 0.25 | 0.5 | 0 | 0.125 | 0.25 | 0.5 |    |      |
| No. of animals   | 5    | 5  | 5  | 5  | 5 | 5  | 5  | 5  | 5  | 5    |

Injection site

-Cell infiltration, inflammatory, focal ± 1 0 0 0 ± 0 1 0 0
-Cell infiltration, macrophages, focal ± 0 1 0 0

Grade- ±, minimal.

single-dose toxicity test of BVP in SD rats to determine an appropriate dosage for its safe use. The results showed no treatment-related abnormalities for any of the used doses of BVP. The dose used for the high-dosage group was 0.5 mL/animal, and no dangerous signs were observed. Thus, we may conclude that 0.5 mL/animal of BVP is a safe dose in both male and female SD rats.

5. Conclusion

This study showed that the lethal dose of BVP was over 0.5 mL/animal in both male and female SD rats.

Conflict of interest

The authors declare that there are no conflict of interest.

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