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RESEARCH ARTICLE

A Toxicological Study of HangAmDan-B in Mice

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Abstract
The aim of the study was to define the toxicity of HangAmDan-B (HAD-B) in mice over the short and long term. HAD-B was studied in 1-week single and 5-week repeated oral dose toxicity tests on male Imprinting Control Region mice. Doses used in 1 week single oral dose toxicity tests were 0, 0.2, 1, 5, and 25 g/kg/day and those of repeated toxicity test were 0, 0.04, 0.2, 1, and 2 g/kg/day. Blood and urine samples were assayed and their morphology observed. Numerical data were compared using Mann-Whitney U test and analysis of variance. Significantly decreased red blood cell levels in mice from S2-HAD-B, S3-HAD-B, S4-HAD-B, and S5-HAD-B groups were observed in single oral dose toxicity tests. Hemoglobin, hematocrit, and mean cell hemoglobin values in mice from the S4-HAD-B and S5-HAD-B groups were also significantly decreased. No mortalities or significant differences in all factors were observed during the dosing period of the repeated dose toxicity test. Administering 2 g/kg/day of HAD-B in mice over a 5-week period showed no significant hematological changes. However, risk of anemia with more than 5 g/kg/day administration of HAD-B was found. In general, HAD-B appears to be safe and nontoxic, and a no observed adverse effect level in mice was established at 2 g/kg/day. This data serves as satisfactory preclinical evidence for the safety of HAD-B should a future clinical trial for HAD-B be launched. Further studies are required to confirm these safety results and to carry out a safety trial in humans.

1. Introduction

HangAmDan-B (HAD-B) is an upgraded version of HangAmDan (HAD) which has been used for solid tumors such as pancreatic, lung, colorectal, and stomach cancers [1–6] at the East West Cancer Center (EWCC), Dunsan Oriental Hospital, Daejeon, South Korea, since its development in 1996.

HAD comes from "Xi Huang Wan," a formula for solid masses, mentioned in the classical text *Wai Ke Zheng Zhi Quan Sheng Ji* (by Pan Wei, 1883). From the original ingredients of Xi Huang Wan, modifications have been made through multiple screening of herbs and research. As a result, HAD was developed consisting of *Coicis semen, Panax notoginseng Radix, Hippocampus, Cordyceps militaris,*

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Cremastrae appendiculata Tuber, Panax ginseng Radix, Bovis calculus, Margarita, and *Moschus*. Several research findings have supported the efficacy of HAD in immune function, anti-angiogenesis, and inhibition of cancer cell proliferation and metastasis [7–9]. Case reports have even been selected as part of the National Cancer Institute's Best Case Series Program using HAD [10]. However, complications with future utilization of HAD have appeared. *Hippocampus* was selected as one of the Convention on International Trade in Endangered Species' endangered animals [11] and the high quantity of *Coicis semen* required for the formula, with its relatively low anti-cancer effect and high starch content, was seen inappropriate for long term usages. To overcome these difficulties HAD-B has been newly formulated by removing *Coicis semen, Hippocampus,* and *Moschus* and by adding *Boswellia carteri* and *Commiphora myrrha*. According to Oriental medicine, *Boswellia carteri* and *Commiphora myrrha* have the ability to eliminate pathological masses, promote blood circulation, remove blood stasis, and control pain. After the modification, HAD-B has shown better efficacy in inhibiting migration and proliferation of human umbilical vein endothelial cells and restraining formation of capillary tube structures when compared with HAD [12]. Nonetheless, study and evaluation on safety of HAD-B remains minimal because of its recent development.

The necessity of defining the safety of HAD-B for clinical use has increased and is inevitable for future, broader application of HAD-B in oriental medicine cancer therapy. In this study, toxicity of HAD-B as a whole formula is investigated rather than individual herbal ingredients; given that in clinical settings, use of multiple herbs as a prescription is far more common, although the exact chemical interactions of each herb are as yet unknown.

Systemic clinical toxicity studies of the formula have not been performed due to the limitations of conducting a human clinical trial using a prescription without any evidence of safety. Therefore, preliminary studies to evaluate the safety of HAD-B in 1-week single and 5-week repeated oral dose toxicity tests to build safety evidence for future clinical trials are conducted in these investigations.

2. Materials and Methods

2.1. Test materials

HAD-B is an herbal formula consisting of eight herbal ingredients in a powder form (Table 1). The crude ground herbs of HAD-B were obtained from Dunsan Oriental Hospital (Daejeon, Korea), and stored at −20°C until use.

Table 1 Ingredients of HangAmDan-B

Herbs (Latin botanical name)	Relative amount (mg)
Panax Notoginseng Radix	95.2
Cordyceps Militaris	71.4
Cremastrae appendiculata Tuber	71.4
Panax ginseng Radix	71.4
Bovis Calculus	47.6
Margarita	47.6
Boswellia carteri	47.6
Commiphora myrrha	47.6
Total amount (1 capsule)	499.8

2.2. Animals

Fifty male Imprinting Control Region (ICR) mice (30–32 g, 5-week old upon receipt, Samtako, Korea) were used after 8 days of acclimatization. Animals were housed individually in suspended wire cages (500 mm × 300 mm × 200 mm) in a temperature (20–25°C) and humidity (40–45%) controlled room. Light/dark cycle was 12/12 hours and feed (Pellet, Samyang, Korea) and water were supplied free to access during the study.

2.3. Toxicity study

No observed adverse effect level was sought for mice. Fifty male ICR mice were equally distributed into 10 groups (5 per each of the 5 groups for single dose toxicity testing, 5 per each of the 5 groups for repeated dose toxicity testing). Based on the use of HAD-B in animal studies and clinical applications, dosing levels of 0, 0.2, 1, 5 and 25 g/kg/day for single dose and 0, 0.04, 0.2, 1 and 2 g/kg/day for repeated dose toxicity tests were selected.

Representative dose preparations for each level were analyzed for homogeneity of distribution, concentration, and stability during the study. An appropriate amount of the test or vehicle control substance was administered orally to each mouse for 1 week in the single dose toxicity test and for 5 weeks in the repeated dose toxicity test. The 5-week period for longer term repeated dose toxicity test was selected based on EWCC's standard hospitalization period of 3 weeks, giving 2 weeks before and after the treatment time. Mice were observed for mortality, signs of gross toxicity, and behavioral changes at least once a day during the study.

Body weights were recorded during the acclimation period and weekly during the exposure period. Ophthalmological examination was conducted by observing the manifestation of both eyes on the last week of the experiment. Blood was collected from all mice at the end of the study prior to sacrifice.

Blood samples were evaluated for clinical pathology findings.

2.3.1. Single oral dose toxicity test

Single oral dose toxicity testing was conducted on five groups of mice (0 g/kg, S1-HAD-B; 0.2 g/kg, S2-HAD-B; 1 g/kg, S3-HAD-B; 5 g/kg, S4-HAD-B; 25 g/kg, S5-HAD-B) for 1 week. While the extract was delivered via oral Zonde needle ϕ 0.9 × 50 mm (Natsume Seisakusho Co., Ltd., Tokyo, Japan) to test groups, distilled water was administered to the control group. Total volume administered was 8.8 μL.

2.3.2. Repeated oral dose toxicity test

Repeated oral dose toxicity testing was conducted on five groups of mice (0 mg/kg, R1-HAD-B; 0.04 g/kg, R2-HAD-B; 0.2 g/kg, R3-HAD-B; 1 g/kg, R4-HAD-B; 2 g/kg, R5-HAD-B) for 5 weeks. While the extract was delivered via oral Zonde needle ϕ 0.9 × 50 mm (Natsume Seisakusho Co., Ltd., Tokyo, Japan) to test groups, distilled water was administered to the control group. Total volume administered was 8.8 μL.

2.4. Hematology, biochemical parameters and urine analysis

All mice were fasted approximately 18 hours prior to each blood draw. Blood samples were collected from the descending aorta under ether anesthesia at the end of the experiment. Urine analysis for specific gravity, pH, leukocyte, nitrite, protein, glucose, ketone, urobilinogen, bilirubin, and blood was conducted using Bayer Diagnostics Multistix 10SG reagent strips (Bayer Inc., Pittsburgh, PA, USA) and urine chemistry analyzer Clinitek 500 (Siemens Inc., DC, USA).

2.5. Morphologic pathology evaluation

In repeated toxicity testing, complete morphologic evaluations were performed immediately following the death of the animals to avoid autolysis of the organs. Fasted surviving animals were weighed before sacrifice at the end of the trial using anesthetic ether.

Gross pathologic evaluations were performed and weights of the liver, kidney, heart, spleen, lung, testis, and brain were measured and recorded immediately afterwards. Relative organ weight (organ to body weight ratio) were also calculated and recorded.

2.6. Statistical analysis

Mann-Whitney U test was used to compare the homogeneity of variance in numerical data. One way analysis of variance was conducted when homogeneity of variance in data between groups was present. For all analyses, SPSS version 10.1 (SPSS Inc., Chicago, IL, USA) was used and a p value of less than 0.05 was considered statistically significant.

3. Results

3.1. S2-, S3-, S4- and S5-HAD-B groups showed abnormal hematologic values

Comparatively lower red blood cell (RBC) levels were seen in S2-HAD-B (8.5 M/μL), S3-HAD-B (8.7 M/μL), S4-HAD-B (8.5 M/μL), and S5-HAD-B (8.3 M/μL), which were statistically significant from control. Hemoglobin (Hb) levels in S2-HAD-B (11.8 g/dL) and S3-HAD-B (12.0 g/dL) were higher than that of S1-HAD-B and significantly higher than that of S4-HAD-B (8.7 g/dL) and S5-HAD-B (8.3 g/dL). Hematocrit (HCT) levels in S2-HAD-B (39.0%), S4-HAD-B (37.3%), and S5-HAD-B (37.4%) were significantly lower than that of S1-HAD-B. Mean cell hemoglobin (MCH) was higher in S2-HAD-B (14.0 pg) and S3-HAD-B (13.7 pg) than that of S1-HAD-B. In contrast, MCH levels of S4-HAD-B (10.3 pg) and S5-HAD-B (9.9 pg) were lower than that of S1-HAD-B (Table 2).

3.2. S2-, S3-, S4-, and S5-HAD-B groups showed abnormal values of total protein, albumin, BUN, creatinine and triglycerides

Lower levels of total protein were shown in S2v-HAD-B (4.7 g/dL), S3-HAD-B (4.7 g/dL), S4-HAD-B (4.7 g/dL), and S5-HAD-B (4.7 g/dL). The A/G ratio levels of S3-HAD-B (1.1) and S5-HAD-B (1.1) were statistically significant. Creatinine levels of S2-HAD-B (0.1 mg/dL), S3-HAD-B (0.1 mg/dL), S4-HAD-B (0.1 mg/dL), and S5-HAD-B (0.2 mg/dL) were all lower than that of S1-HAD-B. BUN levels of S2-HAD-B (26.9 mg/dL) and S5-HAD-B (25.8 mg/dL) were also lower than that of S1-HAD-B. Triglyceride levels of S2-HAD-B (190.6 mg/dL), S3-HAD-B (245.8 mg/dL), S4-HAD-B (168.2 mg/dL), and S5-HAD-B (163.8 mg/dL) were also significantly lower than that of S1-HAD-B (Table 3).

3.3. R2-HAD-B mice show significant increase in body weight

Average body weight of the treated mice was comparable to that of the control group regardless of dosage level. Notably, we observed significantly higher body weight in the R2-HAD-B group at week 4 compared with the control group (Figure 1).

Table 2 Hematologic values of mice treated with HangAmDan-B (HAD-B) with 1-week oral administration

Group/dose (g/kg/day)	WBC (K/μL)	RBC (M/μL)	Hb (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	PLT (K/μL)
S1-HAD-B (0)								
Mean	1.0	9.9	10.4	44.5	45.0	10.6	23.5	820.0
SD	0.17	0.48	0.47	2.12	0.23	0.08	0.11	81.39
No.	5	5	5	5	5	5	5	5
S2-HAD-B (0.2)								
Mean	0.8	8.5*	11.8*	39.0*	46.0	14.0*	30.3*	848.2
SD	0.13	0.24	0.34	0.98	0.56	0.35	0.47	40.82
No.	5	5	5	5	5	5	5	5
S3-HAD-B (1)								
Mean	1.3	8.7*	12.0*	39.9	45.8	13.7*	30.0*	863.0
SD	0.28	0.26	0.27	1.20	0.56	0.19	0.48	19.05
No.	5	5	5	5	5	5	5	5
S4-HAD-B (5)								
Mean	1.2	8.5*	8.7*	37.3*	44.0*	10.3*	23.4	995.4
SD	0.23	0.21	0.23	0.90	0.33	0.09	0.33	47.00
No.	5	5	5	5	5	5	5	5
S5-HAD-B (25)								
Mean	1.1	8.3*	8.3*	37.4*	44.9	9.9*	22.2*	942.0
SD	0.07	0.15	0.18	0.98	0.51	0.17	0.44	20.21
No.	5	5	5	5	5	5	5	5

*$p<0.05$. WBC=white blood cells; RBC=red blood cells; Hb=hemoglobin; HCT=hematocrit; MCV=mean corpuscular volume; MCH=mean cell hemoglobin; MCHC=mean corpuscular hemoglobin concentrations; PLT=platelet.

Table 3 Urinalysis of mice treated with HangAmDan-B (HAD-B), 1-week oral administration

Group/dose (g/kg/day)	Total protein (g/dL)	Albumin (g/dL)	A/G ratio	Creatinine (mg/dL)	BUN (mg/dL)	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	GOT (U/L)	GPT (U/L)
S1-HAD-B (0)									
Mean	5.1	2.5	1.0	0.3	33.8	90.8	326.6	73.8	48.8
SD	0.10	0.05	0.02	0.02	1.43	4.45	23.08	9.97	12.08
No.	5	5	5	5	5	5	5	5	5
S2-HAD-B (0.2)									
Mean	4.7*	2.4*	1.0	0.1*	26.9*	88.2	190.6*	71.0	35.2
SD	0.04	0.04	0.02	0.00	0.90	3.80	9.18	10.56	1.47
No.	5	5	5	5	5	5	5	5	5
S3-HAD-B (1)									
Mean	4.7*	2.2	1.1*	0.1*	32.2	80.6	245.8*	131.6*	56.8
SD	0.12	0.07	0.02	0.02	2.69	4.34	20.72	25.93	20.73
No.	5	5	5	5	5	5	5	5	5
S4-HAD-B (5)									
Mean	4.7*	2.4	1.1	0.1*	33.3	84.4	168.2*	75.8	38.6
SD	0.04	0.08	0.08	0.02	1.35	3.51	10.61	4.57	5.25
No.	5	5	5	5	5	5	5	5	5
S5-HAD-B (25)									
Mean	4.7*	2.5	1.1*	0.2*	25.8*	90.2	163.8*	66.0	33.8
SD	0.04	0.03	0.02	0.05	1.43	3.80	16.68	4.95	3.01
No.	5	5	5	5	5	5	5	5	5

*$p<0.05$. A/G=albumin/globulin; BUN=blood urea nitrogen; GOT=glutamic-oxaloacetic transaminase; GPT=glutamic-pyruvic transaminase.

3.4. R2-, R3-, and R4-HAD-B groups showed MCV and MCHC

The abnormal mean corpuscular volumes (MCV) of R2-HAD-B (42.8 fL) and R3-HAD-B (43.0 fL) were higher than that of R1-HAD-B. Mean corpuscular hemoglobin concentrations (MCHC) of R2-HAD-B (29.5 g/dL) and R4-HAD-B (29.7 g/dL) were also lower than that of R1-HAD-B (Table 4).

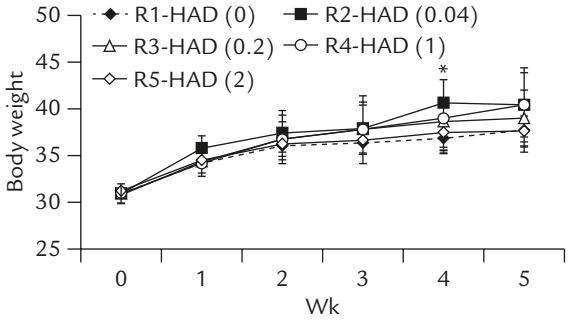

Figure 1 Body weight changes of mice in 5-week repeated oral dose toxicity test for HangAmDan-B (HAD-B). Average body weight of R2-HAD-B group mice was significantly increased after 4 weeks of administration of HAD-B (0 g/kg, S1-HAD-B; 0.2 g/kg, S2-HAD-B; 1 g/kg, S3-HAD-B; 5 g/kg, S4-HAD-B; 25 g/kg, S5-HAD-B).

3.5. R2- and R4-HAD-B groups have abnormal creatinine, BUN and A/G ratio values

The creatinine level of R2-HAD-B (1.1) was significantly lower than that of R1-HAD-B. The blood urea nitrogen (BUN) level of R2-HAD-B (25.7 mg/dL) was lower than that of R1-HAD-B. The albumin/globulin (A/G) ratio of R4-HAD-B (1.1) was significantly higher than that of R1-HAD-B (Table 5).

4. Discussion

This study was performed to determine the toxicity of HAD-B in short term and long term settings. *Boswellia carteri* and *Commiphora myrrha* are two new herbs that have been added to the previously used anti-cancer formula HAD of the EWCC, Korea. Both herbs are known to have actions to remove pathological masses and blood stasis, promote blood circulation, control pain, and inhibit angiogenesis. However, studies and evaluation on safety of HAD-B are minimal due to its recent development.

Although studies on toxicity of HAD-B are limited, there are studies reporting toxicities of the single herbs used in HAD-B. The LD_{50} for ginseng ranges from 10 to 30 g/kg, with a lethal oral dose

Table 4 Hematologic values of mice treated with HangAmDan-B (HAD-B), 5-week oral administration

Group/dose (g/kg/day)	WBC (K/μL)	RBC (M/μL)	Hb (g/dL)	HCT (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	PLT (K/μL)
R1-HAD-B (0)								
Mean	1.5	8.6	11.0	35.6	41.3	12.7	30.8	823.0
SD	0.19	0.09	0.24	0.45	0.25	0.14	0.40	22.91
No.	5	5	5	5	5	5	5	5
R2-HAD-B (0.04)								
Mean	1.6	8.6	10.8	36.7	42.8*	12.6	29.5*	844.6
SD	0.28	0.13	0.14	0.41	0.46	0.19	0.23	30.85
No.	5	5	5	5	5	5	5	5
R3-HAD-B (0.2)								
Mean	1.5	8.6	11.0	37.0	43.0*	12.8	29.8	814.4
SD	0.29	0.18	0.07	0.88	0.50	0.22	0.60	31.98
No.	5	5	5	5	5	5	5	5
R4-HAD-B (1)								
Mean	1.9	8.8	10.9	36.8	41.7	12.4	29.7*	871.2
SD	0.54	0.18	0.15	0.69	0.21	0.13	0.32	26.32
No.	5	5	5	5	5	5	5	5
R5-HAD-B (2)								
Mean	1.7	8.8	10.9	36.2	41.1	12.4	30.0	859.0
SD	0.33	0.17	0.36	0.82	0.42	0.20	0.42	16.47
No.	5	5	5	5	5	5	5	5

*$p<0.05$. WBC=white blood cells; RBC=red blood cells; Hb=hemoglobin; HCT=hematocrit; MCV=mean corpuscular volume; MCH=mean cell hemoglobin; MCHC=mean corpuscular hemoglobin concentrations; PLT=platelet.

Table 5 Urinalysis of mice treated with HangAmDan-B (HAD-B), 5-week oral administration

Group/dose (g/kg/day)	Total protein (g/dL)	Albumin (g/dL)	A/G ratio	Creatinine (mg/dL)	BUN (mg/dL)	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	GOT (U/L)	GPT (U/L)
R1-HAD-B (0)									
Mean	4.8	2.4	1.0	0.2	32.2	74.6	279.8	66.2	33.4
SD	0.15	0.09	0.03	0.04	2.36	7.17	53.85	8.28	5.84
No.	5	5	5	5	5	5	5	5	5
R2-HAD-B (0.04)									
Mean	4.6	2.3	1.0	0.1*	25.7*	74.4	183.8	80.8	63.4
SD	0.04	0.04	0.04	0.00	0.94	3.83	20.38	25.06	36.81
No.	5	5	5	5	5	5	5	5	5
R3-HAD-B (0.2)									
Mean	4.6	2.2	1.1	0.2	32.1	71.0	212.0	122.4	66.0
SD	0.09	0.05	0.05	0.04	1.60	2.12	11.00	29.56	18.88
No.	5	5	5	5	5	5	5	5	5
R4-HAD-B (1)									
Mean	4.7	2.5	1.1*	0.2	32.5	71.8	234.4	67.8	40.4
SD	0.14	0.11	0.05	0.03	1.29	5.24	13.29	7.74	4.67
No.	5	5	5	5	5	5	5	5	5
R5-HAD-B (2)									
Mean	5.0	2.6	1.1	0.2	34.4	75.2	264.2	70.0	30.6
SD	0.07	0.04	0.03	0.03	1.51	2.13	33.61	9.33	2.56
No.	5	5	5	5	5	5	5	5	5

*$p < 0.05$. A/G=albumin/globulin; BUN=blood urea nitrogen; GOT=glutamic-oxaloacetic transaminase; GPT=glutamic-pyruvic transaminase.

set as high as 5 g/kg in mice [13]. In humans, 100 mL of 3% ginseng extract intake will cause light anxiety and 200 mL of 3% ginseng extract intake will bring on itchiness and headache [14]. According to a study, suggested clinical dosage of ginseng is 2.0 to 10 g/day with adverse effects of hypertension, gastrointestinal disturbances, insomnia, and nervousness when taken beyond the safe dosage [15]. Another study suggests clinical dosage of *Cremastae appendicular Tuber* to be 3–10 g/day, and mentions adverse effects of gastrointestinal disorder such as nausea and vomiting [16].

HAD-B was tested in 1-week single and 5-week repeated oral dose toxicity tests on male ICR mice. In single oral dose toxicity tests, no mortalities, no ophthalmologic findings, and no abnormal clinical signs were observed. There were no significant differences in average body and organ weights between control and treated groups regardless of dosage level. However, in hematological analysis, significantly lower RBC values in mice of the S2-HAD-B, S3-HAD-B, S4-HAD-B, S5-HAD-B were observed. Hb, HCT, and MCH values in mice of the S4-HAD-B and S5-HAD-B groups were also significantly low. Lower levels of RBC, Hb, HCT and MCH indicate blood loss, which may be caused by gastrointestinal bleeding, bleeding in internal organs, and hemolytic anemia. In this case, hemolytic anemia was ruled out as urinalysis results revealed no presence of free hemoglobin in urine samples. More tests are needed for differential diagnosis such as occult blood test for gastrointestinal bleeding and autopsy to determine whether the internal bleeding was present or not. Special attention is recommended when administering HAD-B to patients with gastric ulcer or anemia, considering the possible risk of blood loss with more than 5 g/kg/day of HAD-B intake.

Overall values of biochemical parameters indicated that treated mice were comparable to controls regardless of dosage level. However, S2-HAD-B, S3-HAD-B, S4-HAD-B, and S5-HAD-B groups showed significant decrease in creatinine and triglyceride levels. Creatinine level decrease when body muscle mass is reduced. However, all mice gained body weight; thus, the decreased creatinine level could not be explained in terms of weight changes. Triglycerides can induce atherosclerosis when increased. Decreased triglycerides indicate an improved metabolic condition in the body. No significant differences between the control group and treatment groups were found during the dosing period in urinalysis assessment.

In repeated dose toxicity testing, there were no mortalities, no ophthalmologic findings, and no abnormal clinical observations made. Average overall body weight data indicated that the treated mice were comparable to the controls regardless of dosage level. Hematological analysis showed no differences in most parameters examined. The average overall values for biochemical parameters indicated that the treated mice were comparable to controls regardless of dosage level. There were no significant differences in urine analysis during the dosing period between all groups.

Administering 2 g/kg/day of HAD-B in mice over a 5-week period showed no significant hematological changes. However, risk of anemia with more than 5 g/kg/day of HAD-B administration was found. In general, HAD-B appeared to be safe and non-toxic according to the findings, and a no observed adverse effect level in mice was established at 2 g/kg/day. This data serves as satisfactory preclinical evidence of safety to allow the launch of future clinical trials for HAD-B. More studies are required to confirm these safety results and to further carry out a safety trial in humans.

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