Long-term Toxicity Tests of the Effect of OSTEOKING on Laboratory Rats

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1. Purposes of the Test

In compliance with Appendix 5 of *Revision and Complementary Stipulations Concerning Chinese Drugs: Technical Requirements for Toxicological Research* (September 1992), we undertook long-term toxicity tests and research on rats to assess treatment with OSTEOKING. The purpose was to observe the reactions, toxic symptoms, and toxicity in target organs of these animals after they had received the drugs continuously for 48 days, thereby providing an experimental basis to evaluate the safety of treatment with the medicine.

2. Materials

2.1 Medicine to be tested:

OSTEOKING, sepia solution, 1 ml of the solution equivalent to 0.17g of crude drug. The solution was provided by Yunnan Crystal Natural Drug Pharmaceutical Factory, Kunming, China, Lot No. 980510. The drug solution was dispensed into three different concentrations just before the test, i.e., the clinical concentration and 2 times and 4 times this concentration.

2.2 Testing animals:

SD rats, body weight 121 ±6.8g, male and female in equal proportions, provided by Animal Feeding Room, the Key Laboratory of Natural Drugs Pharmacology of Yunnan Province, Animal Quality Certificate No.0000457.

2.3 Testing apparatus and reagents

CEU - DYN 1600, type Full - automatic Blood Cell Counter
(manufactured by Abbott Co. U.S.A)
OLYMPUS - AU 600, type Full - automatic Biochemical Analysis Instrument
(imported from Japan)
Biochemical reagent (provided by Shanghai Changzhen Co. and
Beijing Zhongsheng Co.)

3. Testing Methods

3.1 Design and methods of drug administration:

Animals in the test group were divided into high, medium, and low dosage groups. There were 20 rats in each group, half of them male and half female. Rats in each group were injected with 20ml/kg (body weight) of OSTEOKING in concentrations four times normal, two times normal, and normal for the clinical drug, respectively. As in the clinical trials, the drugs were given once every 4 days for 48 days (or 12 times altogether, the equivalent of 2-4 therapeutic courses of the

clinical drug). A control group was also established with a corresponding animal population. Animals in this group were injected with distilled water in dosages equivalent to the test group. Animals in both groups were weighed once before and once after drug administration, and drug dosage (or distilled water dosage for the control group) was regulated according to changes in body weight.

3.2 Observations and assays

After 48 days of continuous drug administration, blood samples were taken from the femoral arteries, and the animals were sacrificed simultaneously. We then undertook assays of the targets of hematology, hemobiochemistry, histopathology, etc., respectively.

3.3 Statistical method

The test data were handled using the method of statistical analysis of variance.

4. Observations, Results, and Statistical Analyses

4.1 General observation

During the testing period, the appearance, hair color, activity, limb movement, and excrement remained normal except that appetite in the high-dosage group was slightly reduced in the early stages of the test. There were no apparent differences in the changes of body weight between the test group and the control group or among the three drug-concentration groups. The results are shown in Table 1 and Figure 1 below:

Table 1. Increases in rat body weight following injection with OSTEOKING for 48 days ($\bar{x}$ ± SD (g) n=20)

Group		Body weight	Body weight at different times (day)			
			7	14	28	48
Control group		121.3 ± 6.6	139.5 ± 15.5	158.0 ± 15.6	193.7 ± 20	262.2 ± 33.1
Drug-Dosage Groups	Low dosage	119.6 ± 5.9	140.6 ± 11.5	155.0 ± 13.4	197.0 ± 18.7	259.1 ± 32.9
	Medium dosage	120.3 ± 4.2	135.7 ± 7.4	148.6 ± 9.5	194.9 ± 17.1	254.1 ± 26.4
	High dosage	121.7 ± 8.8	140.7 ± 13.4	154.1 ± 13.4	194.4 ± 25.2	247.3 ± 27.5

Comparisons among the three drug-dosage groups and between the test group and control group showed that all p values were greater than 0.05 ($p > 0.05$).

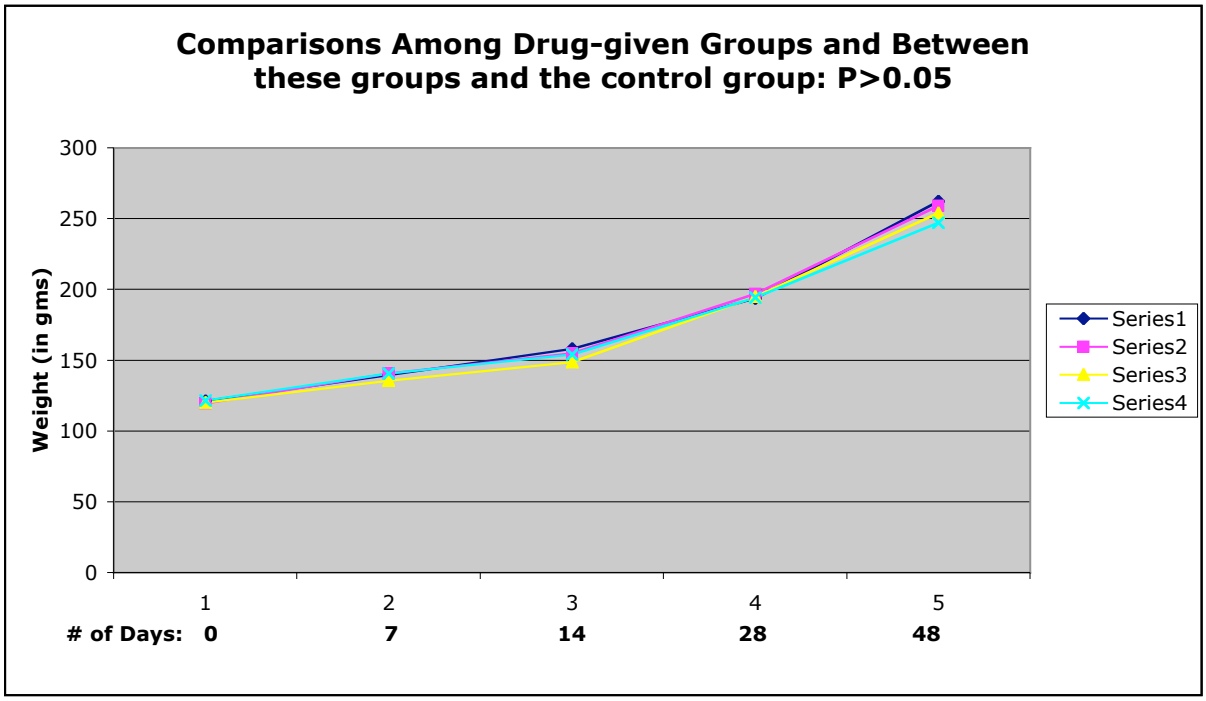

Figure 1: Increases in rat body weight after injection with OSTEOKING for 48 days.

4.2 Assay of hematology and the results.

Hematology assay targets included RBC and WBC counts, classification count, Hb and PLT counts, Ret count, coagulation times test, etc. The results of comparisons among the three drug-dosage groups and between the test group and control group showed no adverse effect of the drug on the hematology of the test animals (Tables 2 and 3).

Table 2. Hematological influences on rats after injection with OSTEOKING for 48 days (X ± SD (g) n=12)

Group		Coagulation time (seconds)	RBCX 10^{12}/L	Hb(g/1)	PLT X 10^9/L	Ret%
Control		76.3 ± 27.5	8.0 ±0.6	163.3 ± 12.2	553.2 ± 160.2	0.9 ± 0.2
Drug Dosage Groups	Low D	85.0 ± 14.8	7.5 ± 0.4	157.8 ± 8.4	485.8 ± 85.4	0.8 ± 0.1
	Medium D	80.0 ± 16.1	7.0 ± 1.1	155.1 ± 6.3	519.3 ± 164.7	1.4 ± 0.3
	High D	78.8 ± 14.5	8.1 ± 0.5	165.1 ± 9.6	503.7 ± 143.1	1.5 ± 0.3

Comparisons among the three drug-dosage groups and between these test groups and the control group showed that P >0.05

			Neutrophil	Lymph	Moyocaryon
Control group		11.0 ± 2.9	31.5 ± 7.5	68.0 ± 7.1	<1
Drug-Dosage Groups	Low D Medium D High D	10.4 ± 3.4 10.6 ± 2.3 9.6 ± 2.2	28.5 ± 5.7 31.2 ± 5.4 29.4 ± 5.5	71.5 ± 5.7 68.9 ± 5.5 76.6 ± 5.5	<2 <1 <1

Table 3: Influences to the targets of hematology of rats which were injected with OSTEOKING for 48 days (X±SD) n=12

Comparisons among the three drug-dosage groups and between these test groups and the control group showed that all *p* values were greater than 0.05 ($p > 0.05$).

4.3 Assays and results of hemobiochemistry:

The observation targets of the hemobiochemistry assay were blood sugar (Glu), glutamic - pyrunic transminase (ALT), glutamic oxaloacetic transminase (AST), alkaline phosphatase staining (AKP), total protein (TP), albumin (ALB), ureal ammonia (BUN), total cholesterol (ChoL), total bilirubin (TB), and creatinine (10 items (see Tables 4 and 5). The results showed there was no apparent effect on the various hemobiochemistry targets after OSTEOKING injection for 48 days. Statistical analysis showed that all *p* values were greater than 0.05 ($p > 0.05$). The assay values were in the normal range for the assayed animals.

Table 4. Hemobiochemistry influences in rats injected with OSTEOKING for 48 days (X ± SD) n=12

Group		ALT(u/L)	AST(u/L)	ALP(u/L)	TB(umol/L)	TP(^L)
Control		71.4 ±12.8	305.1 ± 54.6	291.1 ± 99.0	0.85 ± 0.4	73.1 ± 4.3
Drug-Dosage Groups	Low D Medium D High D	79.0 ± 8.5 72.8 ± 8.1 82.3 ± 10.8	297.0 ± 54.9 295.3 ± 31.1 298.2 ± 30.3	303.4 ± 73.2 299.1 ± 62.8 363.9 ± 74.5	1.2 ± 0.5 0.8 ± 0.4 1.0 ± 0.5	70.4 ± 8.2 68.2 ± 4.7 76.0 ± 3.1

Comparisons among the three drug-dosage groups and between these groups and the control group showed that all *p* values were greater than 0.05 ($p > 0.05$).

Table 5: Hemobiochemistry influence on rats injected with OSTEOKING for 48 days (X ± SD) n=12

Group		ALB(G/L)	BUN (mmol/L)	Cr(umol/L)	Glu(mmol/L)	CHOL (g/L)
Control		46.7 ± 2.9	9.0 ± 2.1	58.0 ± 4.7	5.5 ± 0.9	1.7 ± 0.2
Drug-Dosage Groups	Low D Medium D High D	44.6 ± 3.7 44.7 ± 2.0 49.0 ± 1.7	6.9 ± 1.1 6.8 ± 0.6 7.3 ± 1.3	56.0 ± 4.1 51.6 ± 2.6 54.7 ± 3.7	5.4 ± 0.6 5.4 ± 0.5 5.4 ± 0.7	1.6 ± 0.5 1.6 ± 0.2 1.7 ± 0.3

Comparisons among the three drug-dosage groups and between these groups and the control group showed that all p values were greater than 0.05 ($p > 0.05$).

4.4 Systematic autopsy and histopathology checks

Systematic autopsy and calculations of organ coefficient:
The animals were sacrificed through femoral artery bloodletting when the test was completed. The blood was sent for hematology and hemobiochemistry assays, and we undertook a comprehensive autopsy and careful observation of gross anatomy in the animals, evaluating their hearts, livers, spleens, lungs, kidneys, brains, adrenal glands, testes (ovaries, uteri), prostates, stomachs, duodenums, jejunoileums, colons, thyroids, thymus glands, bladders, lymph nodes, etc. We weighed the important organs and calculated the organ coefficients. No abnormalities were found (see Table 6).

Table 6. Influences on rat organ coefficients after injection with OSTEOKING for 48 days (X ± SDg/l00g of body weight) n=12

Organ	Control group	Low dosage group	Medium dosage group	High dosage group
Heart	0.40 ± 0.05	0.42 ± 0.06	0.39 ± 0.06	0.41 ± 0.06
Liver	3.66 ± 0.28	3.70 ± 0.32	3.61 ± 0.24	3.67 ± 0.33
Spleen	0.34 ± 0.04	0.35 ± 0.04	0.35 ± 0.05	0.34 ± 0.05
Lung	0.64 ± 0.07	0.65 ± 0.06	0.63 ± 0.05	0.64 ± 0.05
Kidney	0.67 ± 0.09	0.68 ± 0.06	0.68 ± 0.08	0.67 ± 0.05
Thymus gland	0.17 ± 0.03	0.16 ± 0.03	0.18 ± 0.04	0.16 ± 0.03
Adrenal gland	0.026 ± 0.006	0.026 ± 0.005	0.026 ± 0.006	0.026 ± 0.006
Testes (n= 10)	1.46 ± 0.12	1.48 ± 0.13	1.49 ± 0.12	1.5 ± 0.16
Prostate (n= 10)	0.72 ± 0.05	0.74 ± 0.08	0.73 ± 0.08	0.74 ± 0.07
Uterus (n=10)	0.35 ± 0.05	0.36 ± 0.06	0.35 ± 0.04	0.35 ± 0.05

Comparisons among the three drug-dosage groups and between these groups and control group showed that all p values were greater than 0.05 ($p > 0.05$)

4.4.2 Histopathology checks:

In accordance with the requirements concerned [2], we undertook the histological checks of the hearts, livers, spleens; lungs, kidneys, adrenal glands, thyroid glands, thymus glands, stomachs, testes (ovaries, uteri), and prostates of half the rats (male and female half and half) selected at random from the control and high-dosage group. (The other two dosage groups were maintained in their original conditions to be checked if any abnormal conditions were found in the high-dosage group). The results were that no histopathological changes related to drug administration were found in either the test group or the control group (Table 7).

Table 7. Results of histopathology checks of various rat organs in long-term toxicity tests of OSTEOKING

Lung	Pneumonectasis	|	|	|	|	|	|	|	|	|	|	|	|	+|	|	|	|	|	|	|	|
	Inflammatory	|	|	|	|	|	|	|	|	|	+|	+|	+|	|	|	|	|	|	|	|	|
	Necrosis	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Bleeding	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Stasis of Blood	|	|	|	|	|	|	|	|	|	|	|	+|	|	|	|	|	|	+|	+|	|
Spleen	Inflamed Lesion	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Necrosis	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Degeneration	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Bleeding	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Stasis of Blood	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Liver	Inflamed Lesion	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Necrosis	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Degeneration	|	|	|	|	|	+|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Bleeding	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Stasis of Blood	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Heart	Inflamed Lesion	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Necrosis	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Degeneration	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Bleeding	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Stasis of Blood	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Thyroid Gland	Pneumonectasis	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Inflammatory	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Necrosis	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Bleeding	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Stasis of Blood	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Adrenal Gland	Inflamed Lesion	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Necrosis	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Degeneration	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Bleeding	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Stasis of Blood	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
Thymus Gland	Inflamed Lesion	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Necrosis	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Degeneration	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Bleeding	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Stasis of Blood	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Sex	M	M	M	M	M	F	F	F	F	F	M	M	M	M	M	F	F	F	F	F
	# of Animal	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10

	Group	Control Group										High Dosage Group									
Kidney	Inflamed Lesion	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Necrosis	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Degeneration	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Bleeding	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Stasis of Blood	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	+|	|	|	|
Uterus	Inflamed Lesion						|	|	|	|	|						|	|	|	|	|
	Necrosis						|	|	|	|	|						|	|	|	|	|
	Degeneration						|	|	|	|	|						|	|	|	|	|
	Bleeding						|	|	|	|	|						|	|	|	|	|
	Stasis of Blood						|	|	|	|	|						|	|	|	|	|
Epididymus	Inflamed Lesion	|	|	|	|	|						|	|	|	|	|					
	Necrosis	|	|	|	|	|						|	|	|	|	|					
	Degeneration	|	|	|	|	|						|	|	|	|	|					
	Bleeding	|	|	|	|	|						|	|	|	|	|					
	Stasis of Blood	|	|	|	|	|						|	|	|	|	|					
Testes	Inflamed Lesion	|	|	|	|	|						|	|	|	|	|					
	Necrosis	|	|	|	|	|						|	|	|	|	|					
	Degeneration	|	|	|	|	|						|	|	|	|	|					
	Bleeding	|	|	|	|	|						|	|	|	|	|					
	Stasis of Blood	|	|	|	|	|						|	|	|	|	|					
Prostate	Inflamed Lesion	|	|	|	|	|						|	|	|	|	|					
	Necrosis	|	|	|	|	|						|	|	|	|	|					
	Degeneration	|	|	|	|	|						|	|	|	|	|					
	Bleeding	|	|	|	|	|						|	|	|	|	|					
	Stasis of Blood	|	|	|	|	|						|	|	|	|	|					
Ovary	Inflamed Lesion						|	|	|	|	|						|	|	|	|	|
	Necrosis						|	|	|	|	|						|	|	|	|	|
	Degeneration						|	|	|	|	|						|	|	|	|	|
	Bleeding						|	|	|	|	|						|	|	|	|	|
	Stasis of Blood						|	|	|	|	|						|	|	|	|	|
Stomach Pancreas	Inflamed Lesion	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Necrosis	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Degeneration	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Bleeding	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Stasis of Blood	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|	|
	Sex	M	M	M	M	M	F	F	F	F	F	M	M	M	M	M	F	F	F	F	F
	# of Animal	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
	Group	Control Group										High Dosage Group									

Notes: (1) "–" means male (positive).

(2) ″ ± " shows a few local, low-grade cellular infiltrations, individual cell degeneration, and one lobe pulmonary emphysema.

(3) "+" shows low-grade stasis of blood and low-grade degeneration.

(4) Since the pathological checks of animals in the control and high dosage group appeared negative, animals in other drug-dosage groups did not undergo pathological checks.

4 Evaluation of results

A long-term toxicity test of OSTEOKING on rats was conducted for 48 days. The high, medium, and low drug-dosage groups reached 16, 8, and 4 times (converted according to surface area) the clinical drug application for adults.

No animal died during the whole test period. When animals were observed outside the containers, no apparent toxic reaction was found.

There were no apparent differences in increases of rat body weight or results of hematological and hemobiochemical checks.

Comparisons among the three drug-dosage groups and between these groups and the control group showed that all the data assayed were in the normal range ($p > 0.05$).

Calculations of the organ coefficient results showed no apparent differences in comparisons among the groups.

We did not find pathological changes in histopathology checks of the important organs of animals selected from the control group and the high-dosage group.

The daily dosage for adults is 50ml, only 1/6 the dosage of animals selected from the high-dosage group (calculated according to surface area). Therefore, it is safe to take the drug continuously according to this dosage.

References

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Original data are kept in Pharmacology Teaching and Researching Section, Pharmacology Department, Kunming Medical College.