

# Application of *Clostridium Botulinum* Toxin Type D to Control Plateau Zokors in Qinghai–Tibet Plateau Pastoral Areas

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**Abstract:** The feasibility of *Clostridium botulinum* toxin type D as a novel biological rodenticide to control plateau zokors was explored. In this study, Horn's method was used to determine the gavage LD<sub>50</sub> of *C. botulinum* toxin type D to plateau zokors. Modified Karber method was employed to determine the oral LD<sub>50</sub> of granular and wheat baits. Results showed that the gavage LD<sub>50</sub> was 5840 MLD/kg (the reliable line is 3430–9950 MLD/kg) and the oral LD<sub>50</sub> values of granular and wheat baits were 0.8339 and 1.319 g/kg, respectively, which indicated that plateau zokors are sensitive to this toxin. The palatability of wheat and homemade granular baits to plateau zokors was measured. Selective feeding coefficients were 1.33 and 1.10, whereas nonselective feeding coefficients were 0.81 and 0.60, respectively. A plot experiment of different toxin concentrations to kill zokors showed that homemade granular bait (10000 MLD/g) had the best effect. The average efficiency rate was 89.83%. This study showed the feasibility of controlling plateau zokors by using botulinum toxin type D.

**Keywords:** *Clostridium Botulinum* Toxin Type D, Rodent Control, Plateau Zokor, LD<sub>50</sub>

## 1. Introduction

The Qinghai–Tibet Plateau pastoral area of China plays an important role in water conservation and global climate regulation [3]. Protecting the source regions of the three major rivers is of great significance to the sustainable socio-economic development and ecological security [4]. However, with the change of the ecological environment and the overuse of grassland resources by humans in recent years, many types of harmful rats, such as *Eospalax fontanierii*, have severely damaged the grassland, and the damage is worsening [5].

*Eospalax fontanierii* is a species of the genus Mole. It spends most of its life in the dark and mainly eats plant roots, especially grassroots underground parts [6]. In recent years,

the population of plateau zokors has increased dramatically. Such increase caused serious negative effects on the ecological area, and the affected area is expanding. This event poses a serious threat to grassland ecological environment and the sustainable development of local livestock husbandry. As reported [7] that the harm area caused by zokors in Qinghai Province was 110 × 104 hm<sup>2</sup>. Yushu, Guoluo, and Huanhu Lake in Qinghai Province were the most endangered areas and formed a large area of “black soil beach.” and the farm rats are found in 130000 hm<sup>2</sup> of Qinghai Province, and approximately 67000 hm<sup>2</sup> harm area is caused by zokors. The largest density is 662 per hm<sup>2</sup>, and the damage is serious [8].

Pest control mainly includes chemical, physical, and biological methods. Rodenticides for rat control are effective in city environments. However, in grassland ecosystems, chemicals can cause secondary poisoning and endanger rodent

predators (eagles, tigers, and foxes) through the food chain; consequently, a sharp decrease in the number of natural predators can eventually dramatically increase the number of rodent populations [9]. The physical control method is performed by capturing or killing mice, thereby reducing the number of rodents. However, this method is not very efficient in the vast prairie. Hence, the chemical and physical methods cannot be used as the main tactics for grassland rodent control.

*Clostridium botulinum* toxin type D has a high application value as a new biological rodenticide to control the density of rodents in plateau areas. In this study, the sensitivity of plateau pika and zokor to *Clostridium botulinum* toxin type D was determined.

## 2. Material

### 2.1. Experimental Agent

Botulinum type D rodenticide (toxicity, 10 million MLD/ml; batch number, 20151003) was provided by the Qinghai Academy for Animal Science and Veterinary Medicine.

### 2.2. Experimental Animals

Plateau zokors were captured from Menyuan and Datong counties, Qinghai Province, by using a utility model, namely, plateau a zokor biopsy capture device.

## 3. Methods

### 3.1. Determination of Gavage $LD_{50}$

In accordance with Horn's method [1], four dose groups of 2150, 4640, 10000, and 21500 MLD/kg, from five animals each group, were designed. The rats were switched to normal feeding and observed for 7 days. The death of the plateau zokors was recorded.

### 3.2. Determination of Oral $LD_{50}$

In accordance with the modified Koji method, five bait groups of 2.0, 1.0, 0.5, 0.25, and 0.125 g bait/200 g were prepared. The bait was placed in a dish. The plateau zokors were feeding alone and, after completely eating the bait, were observed for 7 days. The death of the plateau zokors was recorded.  $LD_{50}$  was calculated by using the modified Koji method [2] (formula:  $LD_{50} = \text{LOG}-1[Xm-i(\sum P-0.5)]$ ).

### 3.3. Determination of Selective Palatability

Five plateau zokors were feeding in a single cage. Two plates, one containing 10 g of bait and another containing 10 g of nontoxic bait, were placed in a breeding cage for the zokors to feed freely. The plates were removed after 12 h, and the unfinished food was weighed. The data were recorded.

### 3.4. Determination of Nonselective Palatability

Ten plateau zokors were randomly divided into two groups. For the first group (poison bait group), plates with 10 g of

wheat poison bait (40000 MLD/g) were placed in each of the five barrels. For the second group (nontoxic group), plates with 10 g of nontoxic wheat were placed in each of the other five barrels. The plates were removed after 12 h, and the unfinished food was weighed. The data were recorded.

### 3.5. Repeatedly Encountered Palatability

Five plateau zokors were feeding in a single cage with bait of sublethal dose. After 24 h, the bait was removed and weighed. After 3 days, 10 g of botulinum toxin type D bait (40000 MLD/ml) was placed in five test barrels. The remaining amount of bait was weighed each after 12 h. The deaths were recorded.

### 3.6. Regional Control Test

#### 3.6.1. Sample Design

The test was divided into five groups: homemade botulinum toxin granular bait (20000 MLD/g), botulinum toxin barley baits (10000, 15000, and 20000 MLD/g), and control group. Each plot areas is 0.25  $\text{hm}^2$  (50 m  $\times$  50 m) were established, and the groups were randomly arranged in the middle with a 50 m-wide protection line set around the periphery.

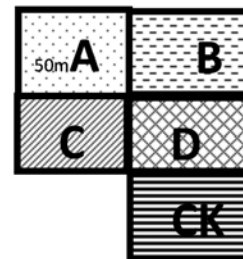


Figure 1. Schematic diagram of community control sample layout.

A: 20000 MLD/g granular bait B: 10000 MLD/g barley bait C: 15000 MLD/g barley bait D: 20000 MLD/g barley bait CK: Control group.

Rat density test: at the start of the experiment, all of the mounds were leveled in the plots, and the data were recorded. After 2 days, the new mound was marked with red chopsticks. The data were recorded, and the rat density in the experimentation area was calculated.

#### 3.6.2. Administration

To ensure the effective rat holes, the feeding tool was penetrated into the cave. Different bait concentrations were placed into the effective hole; each hole contained approximately 10–15 g of bait. The effect was examined after 10 days.

## 4. Results

### 4.1. $LD_{50}$ Test by Oral of Botulinum Toxin Type D to Plateau Zokors

The  $LD_{50}$  of botulinum toxin type D to plateau zokors was 5840 MLD/kg body weight (the reliable line is 3430–9950 MLD/kg [mouse]) (Table 1).

**Table 1.** Determination of LD<sub>50</sub> of botulinum toxin type D to plateau zokors.

Group	Number	Average weight	Dose MLD/kg	Death count	Result
I	5	212.2	2150	0	0/5
II	5	230.6	4640	3	3/5
III	5	208.8	10000	3	3/5
IV	5	254.6	21500	5	5/5

## 4.2. LD<sub>50</sub> of Two Types of Botulinum Toxin Baits to Plateau Zokors by Oral Ingestion

### 4.2.1. LD<sub>50</sub> of Wheat Bait to Plateau Zokors by Oral Ingestion

The LD<sub>50</sub> of the wheat bait containing 40000 MLD/g to plateau zokors was 0.8339/kg weight. This amount indicated that the wheat bait has good palatability to plateau zokors, which are sensitive to the toxin (Table 2 and Table 3).

**Table 2.** LD<sub>50</sub> of wheat bait to plateau zokors by oral ingestion.

Group	Dose (g/200 g)	Number	Average weight (g)	Average dose MLD×10 <sup>4</sup>	Death time (h)	Death ratio
I	1.0	5	181.6	3.64	64 h	5/5
II	0.5	5	201.6	2.02	67 h	5/5
III	0.25	5	173.8	0.87	41 h	3/5
IV	0.125	5	218.4	0.55	72 h	1/5
V	0.0625	5	244.0	0.30	/	0/5

**Table 3.** LD<sub>50</sub> of wheat bait of botulinum toxin type D to plateau zokors.

Group	Dose (g/200 g)	Log (dose)	Number	Death number	Death rate
I	1.0	0.0000000	5	5/5	1.0
II	0.5	-0.3010299	5	5/5	1.0
III	0.25	-0.6020599	5	3/5	0.6
IV	0.125	-0.9030899	5	1/5	0.2
V	0.0625	-1.2041199	5	0/5	0.0

$$LD_{50} = \text{LOG}-1[X_{m-i}(\sum P-0.5)]$$

$$LD_{50} = \text{LOG}-1[-0.3010299-0.9030899(1+0.6+0.2-0.5)] = \text{LOG}-1[-0.3010299-0.9030899(1.3)] = \text{LOG}-1(-1.475) = 0.16879 \text{ (g/200 g)}$$

$$LD_{50} = 0.8339 \text{ g/kg weight}$$

### 4.2.2. LD<sub>50</sub> of Granular Bait to Plateau Zokors

The LD<sub>50</sub> of granular bait containing 40000 MLD/g to plateau zokors was 1.319/kg weight. This amount indicated that the granular bait has good palatability to plateau zokors. (Table 4 and 5).

**Table 4.** LD<sub>50</sub> of granular bait to plateau zokors by oral ingestion.

Group (g/200 g)	Number	Average weight (g)	Average dose MLD×10 <sup>4</sup>	Death time (h)	Death ratio
2.0	5	234	9.36	76 h	5/5
1.0	5	248.6	4.98	104 h	2/5
0.5	5	236.6	2.35	56 h	3/5
0.25	5	316.6	1.59	168 h	1/5
0.125	5	337.2	0.84		0/5

**Table 5.** LD<sub>50</sub> of granular bait of botulinum toxin type D to plateau zokors.

Group	Dose (g/200 g)	Log (dose)	Number	Death number	Death rate
I	2.0	0.301029	5	5/5	1.0
II	1.0	0.0000000	5	2/5	0.4
III	0.5	-0.301029	5	3/5	0.6
IV	0.25	-0.602059	5	1/5	0.2
V	0.125	-0.903089	5	0/5	0.0

$$LD_{50} = \text{LOG}-1[X_{m-i}(\sum P-0.5)]$$

$$LD_{50} = \text{LOG}-1[0.301029-(-0.903089(1+0.4+0.6+0.2-0.5))] = \text{LOG}-1[0.301029-(-0.903089(1.7))] = \text{LOG}-1(-1.475) = 0.26387 \text{ (g/200 g)}$$

$$LD_{50} = 1.319 \text{ g/kg weight}$$

## 4.3. Results of the Nonselective Palatability

The total feeding coefficient was 0.81, which indicated the good nonselective palatability of the wheat bait of botulinum toxin type D to plateau zokors. (Table 6).

**Table 6.** Results of the nonselective palatability test.

Time (h)	First group		Second group		Coefficient
	Consumption (g)	Number of deaths	Consumption (g)		
First day	7.55	0	8.13		0.93
Second day	8.38	0	7.79		1.08
Third day	3.69	1	8.29		0.46
Fourth day	All dead	4	Normal		
Account	19.62	5	24.21		0.81

#### 4.4. Results of the Selective Palatability

The total feeding coefficient was 1.33, which indicated the good selective palatability of the wheat bait of botulinum toxin type D to plateau zokors (Table 7).

**Table 7.** Results of the selective palatability test.

Time (h)	Consumption of poison bait (g)	Consumption of no-poison bait (g)	Feeding coefficient
First day	7.988	5.50	1.45
Second day	4.826	4.42	1.09
Third day	5.83	4.06	1.43
Account	18.64	13.98	1.33

#### 4.5. Results of Repeatedly Encountered Palatability

A sublethal dose of wheat bait was fed to the plateau zokors. The average intakes of each zokor were 5.18, 8.74, and 9.84 g in the each day. No animal death occurred during the trial period.

The food rations were 9.1 and 8.58 g on the first and second

days of feeding with the bait (poison content is 40000 MLD/g), respectively. Three zokors died on the third day, and all of the zokors died on the fourth day. This result indicated that zokors do not exhibit a warning sign or anorexia after the ingestion of a sublethal dose of botulinum toxin type D. Hence, the repeatedly encountered palatability was good.

**Table 8.** Results of the repeatedly encountered palatability.

Group		Number	Weight (g)	Intake (g)				Death time
				First day	Second day	Third day	Fourth day	
Sublethal dose	150 MLD/g Wheat bait	Z01	240	8.8	8.4	10.0		Survived
		Z02	224	2.9	7.9	9.2		Survived
		Z03	313	3.4	10.0	10.0		Survived
		Z04	227	3.2	7.9	10.0		Survived
		Z05	298	7.6	9.5	10.0		Survived
		Z01	240	9.3	9.8	0.7	Dead	72h
		Z02	224	9.8	8.3	Dead		48h
Normal dose	40,000 MLD/g Wheat bait	Z03	313	7.7	7.2	Dead		48h
		Z04	227	8.7	8.2	Dead		48h
		Z05	298	10.0	9.4	8.1	Dead	92h

#### 4.6. Results of Quadrat Sample Experiment

The average killing effect of the granular bait of botulinum toxin type D to the zokors was 89.93%. The average killing effects of the three different concentration groups (0.10%, 0.15%, and 0.20%) were 80.6%, 89.13%, and 87.73%, respectively. In the control group, the average reduction rate was 32.7%. Results showed that the killing effect of the granular bait of botulinum toxin type D was the best among all the baits in this study.

**Table 9.** Killing effect of different concentrations of botulinum toxin type D to control the zokors (0.25 hm<sup>2</sup>).

Group	Repeat	Mound number before control	New mound number	New mound number after control	Efficiency (%)	Mean efficiency (%)
A	I	87	13	0	100.0	89.83
	II	78	9	2	77.8	
	III	73	12	1	91.7	
B	I	57	8	1	87.5	80.06
	II	56	11	3	72.7	
	III	47	10	2	80.0	
C	I	69	9	1	88.9	89.13
	II	75	15	2	86.7	

Group	Repeat	Mound number before control	New mound number	New mound number after control	Efficiency (%)	Mean efficiency (%)
D	III	53	12	1	91.7	87.73
	I	50	8	1	87.5	
	II	68	10	1	90.0	
	III	84	14	2	85.7	
	I	44	11	8	27.3	
CK	II	39	8	5	37.5	32.70
	III	56	12	8	33.3	

Note: A, granular bait; B, 0.10% group; C, 0.15% group; D, 0.02% group.

## 5. Discussion

Since the first isolation of *C. botulinum* in 1897, seven types of *C. botulinum* (i.e., A, B, C, D, E, F, and G) have been discovered [10]. Among these types, A, B, E, and F can cause human botulism; type C causes botulism in cattle, sheep, mink, and poultry; and type G is isolated from the soil, with no reports of poisoning. *Clostridium botulinum* type D causes bovine and mutton poisoning in Africa, North America, and Australia. In China, only two botulinum type D isolations were reported: one was isolated from sea mud [11], and the other was isolated from diseased sheep in our laboratory (Figure 2, Figure 3) [12]. This record shows that botulinum type D is rare in China. Many studies on *C. botulinum* have been reported abroad. However, most of these reports focus [13] on the structure characteristics of the neurotoxin, mechanism of toxin poisoning methods [14, 15]. However, the use of botulinum toxin type D for rodent control has not been reported locally and abroad. The gavage LD50 of *C. botulinum* toxin type D to plateau zokors is 5840 MLD/kg weight (the reliable line is 3430–9950 MLD/kg weight [mouse]). This result showed that the *Myospalax baileyi* is sensitive to botulinum toxin type D. This toxin has ability to control the plateau zokors. Studies have confirmed that the rodenticide has a targeted poisoning effect on the three rodents in the plateau pastoral area, and is safe for environmental animals, including cattle, sheep dogs, and rodent natural enemies such as hawks and vultures [16, 17].



Figure 2. Spores of *Clostridium Botulinum*.



Figure 3. Morphology under electron microscope.

There are many factors influencing the prevention and control of large-scale rodent control, and the palatability of the rodent to the drug is also a key factor. In this experiment, We improved the palatability of rodents on drugs through the improvement of bait formula. The choose palatability is good of wheat bait and the feeding coefficient is 1.33, no choice palatability is good too and the feeding coefficient is 0.81. The experimental results confirmed that the rodent had no obvious rejection of the drug bait, which provided a guarantee for the success of large-scale rodent control.

Combining the results of different concentrations of toxins to kill the plateau zokor and the sensitivity test results of botulinum toxin type D on the plateau zokor, Drawing on relevant research results [18, 19], we recommend using 1.5% concentration of barley poison bait for rodent control in large area rodent control, the effect is better. Excessive drug concentrations pose a potential hazard to environmental animals, but if the drug concentration is too low, it will reduce the effectiveness of rodent control. In addition, it is worth noting that in the process of using D-type botulinum toxin to control plateau zokor, try to avoid the use of oats as a base bait, because the plateau zokor has a peeling effect on oats, otherwise it will seriously affect the effect of extinction.

## 6. Conclusion

Through the sensitivity test of plateau zokor against

botulinum toxin type D rodenticide, palatability test of bait and small area control, combined with the efficacy of D-type botulinum toxin rodenticide on environmental animals and natural enemies of rodents test. It was confirmed that D-type botulinum rodenticide can be used as a safe and efficient rodenticide for the control of rodents in large-scale plateau zokor.

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