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Original Article

Study on a Single-Dose Toxicity Test of *D-Amino Acid Oxidase* (DAAO) Extracts Injected into the Tail Vein of Rats

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Key Words
D-amino acid oxidase, DAO, single-dose toxicity, toxicity test, LD_{50}, injection

Abstract

Objective: This study was performed to analyze the single-dose toxicity of *D-amino acid oxidase* (DAAO) extracts.

Methods: All experiments were conducted at the Korea Testing & Research Institute (KTR), an institution authorized to perform non-clinical studies, under the regulations of Good Laboratory Practice (GLP). Sprague-Dawley rats were chosen for the pilot study. Doses of DAAO extracts, 0.1 to 0.3 cc, were administered to the experimental group, and the same doses of normal saline solution were administered to the control group. This study was conducted under the approval of the Institutional Animal Ethics Committee.

Results: In all 4 groups, no deaths occurred, and the LD_{50} of DAAO extracts administered by IV was over 0.3 ml/kg.

No significant changes in the weight between the control group and the experimental group were observed.

To check for abnormalities in organs and tissues, we used microscopy to examine representative histological sections of each specified organ, the results showed no significant differences in any organs or tissues.

Conclusion: The above findings suggest that treatment with *D-amino acid oxidase* extracts is relatively safe. Further studies on this subject should be conducted to yield more concrete evidence.

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1. Introduction

D-amino acid oxidase (DAAO) is a peroxisomal enzyme containing flavin adenine dinucleotide (FAD) as a cofactor and is in a wide range of species from

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microorganisms to mammals [1]. The enzyme *D-amino acid oxidase* (DAAO) was discovered in the porcine kidney, and since that time, it has been extensively studied as a model flavin-dependent oxidase.

In mammals, DAAO is found at the highest concentrations in the kidneys, liver, and brain. In addition, DAAO catalyzes the oxidative deamination of a wide range of *D-amino acids* [2].

DAAO was first described by Krebs in 1935 [3] and has been found to be one of the most important enzymes for the maintenance of proper levels of *D-amino acids* [4].

The main role of DAAO in mammalian kidneys and liver cells is the detoxification of endogenous *D-amino acids* that accumulate in the organism during the course of racemization. Accumulation of *D-amino acids* in mammalian cells is one of the characteristics of organism aging.

In recent years, the important role of DAAO in maintaining the necessary levels of *D-serine* in different brain tissues has been revealed. *D-serine* participates in the regulation of N-methyl-D-aspartate receptors (NMDA-rs) in the form of a free amino acid or a neuroactive peptide. There have been some suggestions that the dysfunction of NMDA-rs resulting from the erroneous expression of the DAAO gene is one of the possible causes of schizophrenia. The activity of DAAO in malignant kidney and liver cells was also shown to be much lower than in healthy ones, which can be used in the cancer diagnostics of those organs [5].

DAAO plays an important role in regulating the levels of *D-serine,* and its function is impaired by the presence of the *D-serine* mutation, which may contribute to the pathogenic process in Amyotrophic lateral sclerosis (ALS). Sasabe et al. did a study on the role of DAAO and *D-serine* in motorneuron physiology, as well as in ALS pathophysiology, and they showed that *D-serine* homeostasis was physiologically important in motorneuronal excitability and that the inactivity of DAAO was pathologically relevant to the vulnerability of motorneurons to excitotoxicity in ALS. This study also stressed the potential use of regulators of DAAO activity or *D-serine* antagonists as a therapeutic strategy for treating ALS [6].

Taken together, DAAO has potential as a novel therapeutic to treat various neural and psychiatric disorders. However, before clinical experiments can be performed, toxicity tests need to be conducted. Thus, this experiment was conducted to verify the toxicity of DAAO.

The current research trend for single-dose toxicity testing of extracts is to study acute and subacute toxicity through Good Laboratory Practice (GLP). All the experiments for this research were conducted under the GLP at the Korea Testing & Research Institute (KTR), an institution authorized to perform non-clinical studies.

2. Materials and methods

The DAAO (0.1-0.3 cc, Sigma-Aldrich, St. Louis, MS, USA) extract was prepared in a clean room adhering to Korea-Good Manufacturing Practice(K-GMP) in a lab at the Korean Pharmacopuncture Institute. After the mixing process with pure water, the pH was controlled to between 7.25- and 7.35. NaCl was added to make a 0.9% isotonic solution. The completed extract was stored in a refrigerator.

The animals used in this study were 6-week-old Sprague-Dawley rats. The mean weights of the rats were 200.8-233.9 g, and 156.7-183.4 g for the male and female rats, respectively. For all animals, a visual inspection was done and all animals were weighed using a CP3202S system (Sartorius, Germany). After 7 days of acclimatization, the rats' general symptoms and changes in weight were recorded. No abnormalities were found.

The temperature of the lab was 22 ± 3°C and the humidity was 50 ± 20%. Enough food (Cargill Agri Purina) and UV- filtered water were provided.

Groupings were done after 7 days of acclimatization. Animals were selected if their weights were close to the mean weight. In total, 20 male rats and 20 female rats were selected. The animals were distributed into 4 groups (5 mice per group) as follows (Table 1).

The expected dose for *D-amino acid oxidase* extracts was 0.1-0.3 cc, which was determined by "The Study on Acute and Subacute Toxicity and Anti-cancer Effects of Cultivated Wild Ginseng Herbal Acupuncture."[7]. In the control group, the same dose of normal saline solution was administered into a specific point of the tail vein by IV. This study was conducted under the approval of the Institutional Animal Ethic Committee.

On the day of dosing (day 0), the general symptoms (types of toxic symptoms, revealing time, recovering time-,

Table 1 Number of animals

Group	Injection (cc/kg)	Number of animals (serial number)	
		Male	Female
G1 control group	0.3	5 (1101~1105)	5 (2101~2105)
G2 low-dose group	0.1	5 (1201~1205)	5 (2201~2205)
G3 mid-dose group	0.2	5 (1301~1305)	5 (2301~2305)
G4 high-dose group	0.3	5 (1401~1405)	5 (2401~2405)

etc.) and the mortality were examined 30 min, and 1, 2, 3, and 4 h after the injection. From the 1st day to 14th day of treatment, the general symptoms were examined once a day.

The weights were measured immediately before treatment, and at 7 and 14 days after treatment.

After the termination of observation, all surviving animal organs and tissues were visually inspected and examined by microscopy.

The weight results from the experiment were analyzed by using SPSS (version 10.0). Levene's test was conducted to evaluate the homogeneity of the variance and the significance. The One-way ANOVA test was conducted when a homogeneity of the variance was recognized, and the Scheffe's test was conducted post-hoc.

3. Results

In this study, no deaths or abnormalities occurred in any of the groups, and the LD_{50} of the DAAO extracts administered via IV was over 0.3 ml/kg (Table 2, Table 3). In addition, no changes in weight were observed in any of the groups (Table 4). Finally, no meaningful changes in necropsy were noted, and histopathological examination of all of Group 1 (0.3 cc/head) found no significant changes related to injections in the brain, lungs, liver, kidneys and spinal cord (Table 5).

4. Discussion

Paul et al. did a study on the role of *D-amino acids* in amyotrophic lateral sclerosis, pathogenesis, and showed a potential role, such as that of *D-serine* in motor neuron disease/amyotrophic lateral sclerosis (ALS), for *D-amino acids* [8]. D'Aniello et al. did a study on the biological role of DAAO, and showed that the *in vivo* biological role of DAAO in animals is to act as a detoxifying agent to metabolize *D-amino acids* that may have accumulated during aging. If the ingested *D-amino acids* are not metabolized by these enzymes, they will accumulate in the tissues and may provoke serious damage [9].

Smith et al. did a study on the therapeutic potential of DAAO inhibitors. DAAO is a flavoenzyme that degrades *D-amino acids* through the process of oxidative deamination. The physiological role of DAAO in the kidneys and the liver is detoxification of accumulated *D-amino acids*, and increased *D-serine* metabolism resulting from increased DAAO activity may produce a reduction in NMDA receptor activity. The NMDA receptor is thought to play a central role in the pathophysiology of schizophrenia. Taken together, these finding suggest that DAAO inhibitors might be useful as novel therapeutics to treat psychiatric and cognitive disorders [10].

Zhao et al. did a study on the potential role of DAAO in neuropathic pain in a rat model of tight L5/L6 spinal nerve ligation and showed that spinal DAAO contributed significantly to the development of central sensitization-mediated pain, suggesting that DAAO may be an important molecular target for the treatment of chronic pain of neuropathic origin [11]. Verrall et al. did a study on the neurobiology of DAAO, it's involvement in schizophrenia, and the therapeutic value of DAAO inhibition. That study characterized DAAO as an enzyme that degraded the NMDA-R coagonist *D-serine* and that had the potential to modulate NMDA-R function and to contribute to the NMDA-R hypofunction in patients with schizophrenia [12].

Table 2 Mortality

Group	Dose (cc/head)	Mortality (dead / tested) Male	Female
G1	0.3	0%	0%
		0 / 5[a]	0 / 5
G2	0.1	0%	0%
		0 / 5	0 / 5
G3	0.2	0%	0%
		0 / 5	0 / 5
G4	0.3	0%	0%
		0 / 5	0 / 5

[a]: number of dead animals / number of tested animals

Table 3 Clinical signs

Group	Dose (cc/head)	Sex	Number of animals	Clinical signs
G1	0.3	Male	5	NAD
		Female	5	NAD
G2	0.1	Male	5	NAD
		Female	5	NAD
G3	0.2	Male	5	NAD
		Female	5	NAD
G4	0.3	Male	5	NAD
		Female	5	NAD

NAD: no abnormalities detected

Table 4 Body weights in grams

Group	Dose	Sex		Days after administration		
				0	7	14
G1	0.3	Male	Mean	221.9	279.3	331.6
			S. D.	12.9	19.4	26.2
			N	5	5	5
		Female	Mean	169.4	191.7	211.0
			S. D.	2.8	4.0	3.0
			N	5	5	5
G2	0.1	Male	Mean	219.8	285.8	339.9
			S. D.	5.5	13.1	19.3
			N	5	5	5
		Female	Mean	172.2	200.7	225.7
			S. D.	7.5	10.2	9.3
			N	5	5	5
G3	0.2	Male	Mean	219.7	284.9	344.8
			S. D.	6.5	13.5	15.7
			N	5	5	5
		Female	Mean	170.7	201.8	225.6
			S. D.	8.6	14.8	13.6
			N	5	5	5
G4	0.3	Male	Mean	220.4	283.5	334.6
			S. D.	5.5	10.0	10.0
			N	5	5	5
		Female	Mean	170.3	196.0	227.4
			S. D.	8.5	15.0	10.0
			N	5	5	5

N : number of animals, S.D. : standard deviation

Table 5 Necropsy findings

Findings	Group							
	G1 (0.3 cc/head)		G2 (0.1 cc/head)		G3 (0.2 cc/head)		G4 (0.3 cc/head)	
	Male	Female	Male	Female	Male	Female	Male	Female
Number of rats examined	5	5	5	5	5	5	5	5
NGF	5	5	5	5	5	5	5	5

NAD: no abnormalities detected

To assess the toxicity of DAAO, we need to study its acute and chronic harmful effects and its relations with the capacity-reaction more, and animal testing is the most fundamental and basic way to perform safety assessments [13]. The Korea Food & Drug Administration has testing protocol guidelines for the study of toxicity [14], and all experiments should be conducted following Good Laboratory Practice (GLP) regulations.

In this study, the LD_{50} *D-amino acid oxidase* extracts were all about 0.3 cc/head in both male and female rats, which indicates that, compared to those in previous studies, this dose is safe to use and does not cause histological abnormalities.

5. Conclusion

The objective of this study was to analyze the single-dose toxicity of DAAO extracts. All experiments were conducted under the regulations of Good Laboratory Practice (GLP) at the Korea Testing & Research Institute (KTR), an institution authorized to perform non-clinical studies.

The results showed that administration of 0.3-ml/kg DAAO extracts did not cause any changes in weight and did not result in any mortalities which indicates that DAAO administration can be used as a safe treatment.

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