Research on the acute toxicity and teratogenicity of the feeding L-tryptophan

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Abstract. In order to evaluate the safety of the feeding L-tryptophan, we conducted acute toxicity test and teratogenicity test in rats. Rats were fed with L-tryptophan by gavage. The oral LD50 was more than 20.0g/kg b.w. In the teratogenicity test, the doses of L-tryptophan were 5.0g/Kg b.w., 2.5g/Kg b.w. and 1.25g/Kg b.w. We set up a normal control group. The rats were fed by gavage once a day from the 7th to 17th day of pregnancy. At the 20th day of pregnancy, we counted and measured the litter size, placenta weight, live baby weight, body length, tail length, fetus malformation rate, live fetus average malformation rate, maternal fetus malformation rate, fetus death rate, absorptive fetus rate and other indicators. Then we use SPSS12.0 software to carry out data statistical analysis, and One-Way ANOVA and t-test for comparison between groups. The results showed that, when each dose group compared with the control group, there was no significant difference in average body weight, body length, tail length, placenta weight, fetus live rate, fetus death rate and absorptive fetus rate when compared to the normal control group (P>0.05), and there was no significant difference in the common deformity indexes, such as fetus malformation rate, live fetus average malformation rate and maternal fetus malformation rate between each dose group and the normal control group (P>0.05). The oral LD50 of L-tryptophan to Wistar rats was more than 20.0g/kg b.w., and the traditional teratogenicity test was negative, indicating that the feed additive belongs to the actually non-toxic level and has no obvious teratogenicity.

1. Introduction

Tryptophan is an absolutely necessary amino acid for the growth, development and metabolism of animals and human body, which has a variety of physiological functions [1]. L-tryptophan, following after methionine and lysine, is another important amino acid added to feed, which can promote the growth of livestock and poultry, anti-disease and anti-high density feeding [2-4].

L-tryptophan is one of the necessary limiting amino acids in the body, and its synthesis in the body is less, so it needs to be taken from the outside. In vivo, starting from L-tryptophan, 5-hydroxytryptophan, indoleacetic acid, niacin, pigment, alkaloid, coenzyme and other biologically active substance can be synthesized. At present, it has been used as a food additive in many places in
China to enhance the utilization of protein, which plays an important role for fortified food to enhance the utilization of plant protein. In addition, L-tryptophan also have functions of preventing mildew, disinfection and preventing oxidation, which can be used as fish preservative.

L-tryptophan, through blood-brain barrier, can be transformed into 5-Hydroxytryptamine in vivo, and the final product is excreted from urine. In recent years, it has been found that L-tryptophan is not only used for intravenous nutrition infusion, but also has good effects on depression, insomnia, hypertension and pain relief, so it has been used in clinical practice in some European and American countries [5].

L-tryptophan, following after methionine and lysine, is the third largest amino acids added to feed. By adding L-tryptophan, we can adjust the balance of amino acids in the diet and promote the growth of livestock and poultry [6-7]. In addition, the L-tryptophan metabolite, 5-hydroxytryptamine, has the effects of anti high density, weaning and other stress effects in animals, and can enhance the resistance of animals. Because L-tryptophan production cost is gradually reducing, it has been used in pig feed in Japan and Europe and America.

China is a country lacking in protein resources, so it is of great potential value to study how to reduce the protein level of the diet and how to reasonably allocate the low protein diet on the basis of the existing research. On the other hand, the low protein diet can significantly reduce the nitrogen emissions and the odor of excreta in the process of animal production, and reduce the environmental pollution caused by livestock breeding. Therefore, the research and application of tryptophan and other amino acids are of great significance to the conservation of protein resources, the reduction of environmental pollution and the development of livestock breeding. In this paper, L-amino acids produced by a biotechnology company were used to carry out acute toxicity and traditional teratogenicity tests for rats, so as to preliminarily evaluate the application safety of L-amino acids.

2. Materials and methods

2.1. Materials

2.1.1 Drugs and experimental animals

The feeding L-tryptophan, L-tryptophan>20%, it is yellowish granule, the batch number is 20170610. Provided by a biotechnology company.

Preparation of test substance solution: after grinding of test substance L-tryptophan, use 1% sodium carboxymethylcellulose solution to prepare the test substance suspension with.

Wistar rats (clean grade), 10 weeks old, 80 females and 80 males, provided by Yangzhou University Comparative Medicine Center. Animal production license No.: SCXK (Su) 2017-0004. Use license No.: SYXK (Su) 2017-0044. Male and female rats were caged at 1:1. It is the first day of pregnancy when we can see the vaginal plug, which is used in traditional teratogenicity test.

2.1.2 Reagent

Ethanol fixation fluid: 80% ethanol;

Bouin's Fluid: take 750ml of saturated picric acid solution, 250ml of 40% formaldehyde and 50ml of glacial acetic acid, and mix them.

Potassium hydroxide solution: prepare 1% concentration and 2% concentration potassium hydroxide respectively.

Alizarin red stock solution: add alizarin red into the following mixture until it is saturated. Glacial acetic acid 5ml, pure glycerin 10ml, 1% chloral hydrate 60ml.

Alizarin red application solution: take 1ml of Alizarin Red stock solution, add 1% potassium hydroxide solution until 1000ml, and prepare it temporarily before use.

Dehydrated transparent liquid: I glycerol 20mL, 2% Potassium hydroxide solution 3mL, add distilled water to 100mL

II glycerol 50mL, 2% Potassium hydroxide solution 3mL, add distilled water to 100mL
III glycerol 75mL, add distilled water to 100mL.

2.2. Methods

2.2.1 Acute toxicity test
According to the toxicity test process of Han Shiqun [8], the rats were given 5.0 and 15.0g/kg by gavage respectively, and each dose was given to two male and female rats respectively. They did not die within one week. It shows that the toxicity of L-tryptophan in the feed of the tested sample is relatively small, and we can not measure the specific value of LD$_{50}$. According to the technical specification of acute toxicity test, the maximum dose test was carried out for the tested sample. Take 20 rats, half of which are male and half of which are female, and carry out gavage twice within 24 hours with the operable highest concentration and the largest dosage volume (0.8 mL/100g b.w.). The interval of dosing was 6 hours, and the total dosage was 20.0 g/kg b.w.. Observe for 2 weeks and record the poisoning symptoms and death of animals.

2.2.2 Teratogenicity test
(1) Acquisition and treatment of pregnant rats
Cage the female and male rats according to 1:1 and let them stay overnight. The next day, check whether there is a vaginal suppository. The day of discovery of the vaginal suppository is the first day of conception. Weigh, number and randomly group the pregnant rats that day. The experimental doses of L-tryptophan were 5.0g/kg b.w., 2.5g/kg b.w. and 1.25g/kg b.w., and distilled water was used as the normal control group. On the 7th to 17th day of pregnancy, dose them according to 1mL / 100g by gavage once a day, and weigh them on the 1st, 7th, 11th, 15th and 20th day of pregnancy, so as to adjust the dosage and observe the weight change of pregnant rats. Record the pregnant rats at any time. On the 20th day of pregnancy, let the cervical vertebrae of pregnant rats dislocate and die. Immediately cut the abdominal cavity along the midline and take out the uterus. Examine and record the number of corpus luteum on the left and right, the litter weight (conjoined rats in uterus), the weight of uterus, the number of live fetus, the number of dead fetus and the number of absorbed fetus.

(2) Fetal rat measurement and appearance abnormality examination
Cleaned the fetus, weigh the live fetus’s body weight and placenta weight one by one, measure the body length and tail length, and identify the gender. Then we examined the appearance of fetal rat. Pay attention to the location, color, shape, size, quantity of each anatomical part and whether there are abnormal incisions or grooves.

2.2.3 Examination of skeletal deformity in fetal rats
First, make bone samples: put 2/3 of the fetal rats to be tested in 75%~90% ethanol, and fix them for more than 48 hours. Transfer fetal rats into 1% potassium hydroxide solution for 2~7d, during which we change the solution every 1~2d. After the invasion, take out the fetal rats and staine them. Transfer fetal rats into alizarin red solution for 2~3d, shake them 2-3 times a day, and dye their bones red. The colored specimens were transferred into the dehydrated transparent liquid I, II and III for 1d respectively. At this time, the color on the soft tissue gradually faded, and the bone was bright dark red.

Bone examination: pour the specimen and transparent solution into the plate, first observe the whole-body bone with the dissecting microscope, and then carry out the local examination for the abnormal parts with a larger magnification.

2.2.4 Examination of internal organ malformation in fetal rats
Put 1/3 of the fetal rats to be examined in Bouin's Fluid, fix them for 10-15 days, wash them with tap water, put them on the wax plate, cut off the limbs and tail, slice each part of the fetal rats with a blade, and examine the internal organs of the fetal rats.
2.2.5 Calculation of deformity index
Calculate the total number of malformation fetus, the total number of deformities, the average deformity rate of live fetuses and the maternal deformity rate according to different dose groups. When we calculate the total number of malformations, each living fetus is recorded as a malformation fetus as long as it has one or more malformations. When we calculate the total number of malformations, the corresponding number should be included in the total number if a live fetus shows several kinds of malformations.

2.2.6 Data analysis
In this test, the data are expressed as mean± standard deviation. We used SPSS12.0 software for data statistical analysis, and use One-Way ANOVA and t-test for group comparison (test level α=0.05).

3. Result

3.1. Acute toxicity test
When the total amount of the feeding L-tryptophan was 0.0 g/kg b.w., no rats died. During the 2-week observation period after dosing, the animal’s mental state was good, the hair color was bright and clean, the movement was free, the breath, appetite and feces were normal, there was no abnormal secretion in the mouth and nose, and we did not find any abnormal reaction related to dosing. After 14 days, all the animals were examined by pathology, and no obvious pathological changes and abnormalities were found in any organ.

The oral LD50 of rats fed with the feeding L-tryptophan was more than 20.0g/kg b.w.

3.2. Teratogenicity test

3.2.1 Weight changes of pregnant rats
The number of pregnant rats in each experimental group was: 11 in the normal group, the low dose group and the high dose group respectively, and 10 in the middle dose group. The behavior of pregnant rats during pregnancy was normal, without poisoning or death. Comparing the total average weight gain of each group, we can see that there is no significant difference between the pregnant rats in the experimental group and the control group (Table 1).

| Dose group  | Number of pregnant rats | Weight gain of pregnant rats | Total average weight gain |
|-------------|-------------------------|------------------------------|---------------------------|
|             | 1-6days | 7-11days | 12-15days | 16-20days | Total average weight gain |
| Normal control | 11 | 2.18±0.68 | 3.55±0.75 | 1.66±2.66 | 7.07±2.03 | 3.64±0.69 |
| Low dose group | 11 | 2.70±0.72 | 2.11±1.46* | 3.30±1.36 | 4.22±2.17* | 3.05±0.58 |
| Medium dose group | 10 | 2.10±1.38 | 2.88±1.18 | 4.38±0.83* | 4.88±1.74 | 3.45±0.75 |
| High dose group | 11 | 0.62±1.94* | 4.93±4.09 | 5.36±1.38* | 6.58±1.55 | 3.76±0.83 |

Note: In the same row values mark with “*” superscripts mean there has significant difference when compare with the control group (p<0.05)
3.3. Effect on reproductive function of rats
In Table 2, the mean litter weight of the high dose group is significantly better than that of the normal control group. The number of corpus luteum, number of implantation and number of live litter in low dose group and high dose group are significantly better than those in control group ($P<0.05$). There is no statistical difference in the average litter weight, number of corpus luteum, number of implantation and number of live litter between the medium dose group and the normal control group, and there is no statistical difference in the number of absorbed fetus between each dose group and the normal control group ($P>0.05$).

Table 2. Effects of the feeding L-tryptophan on the reproductive function of rats

| Dose group          | Number of pregnant rats | Litter weight (g) | The number of corpus luteum | Implantation number | Number of live litters | Number of absorbed fetus |
|---------------------|-------------------------|-------------------|-----------------------------|---------------------|------------------------|-------------------------|
| Normal control      | 11                      | 53.23±10.16       | 9.55±1.31                   | 9.00±1.64           | 8.64±1.92              | 0.36±0.46               |
| Low dose group      | 11                      | 56.41±5.78        | 11.09±1.39*                 | 10.82±1.22*         | 10.36±1.19*            | 0.45±0.58               |
| Medium dose group   | 10                      | 48.54±11.12       | 10.10±1.30                  | 10.00±1.20          | 9.80±1.40              | 0.20±0.32               |
| High dose group     | 11                      | 61.63±5.98*       | 11.18±0.96*                 | 11.27±1.07*         | 10.82±1.07*            | 0.27±0.40               |

Note: In the same row values mark with "*" superscripts mean there has significant difference when compare with the control group ($p<0.05$).

3.4. Toxicity to rat embryos
In Table 3 and table 4, there is no significant difference in the number of dead fetus, the number of absorbed fetus, absorbed fetus rate, fetus death rate, fetus live rate, the number of litter, the litter average body weight, body length, tail length and placental weight between each dose group and the normal control group ($P>0.05$).

Table 3. Toxicity of the feeding L-tryptophan to the rat embryos

| Dose group    | Number of pregnant rats | Total litter number | Number of live litters | Number of death litter | Number of absorbed fetus | Absorbed fetus rate % | Fetus death rate % | Fetus live rate % |
|---------------|-------------------------|---------------------|------------------------|------------------------|-------------------------|----------------------|------------------|------------------|
| Normal control| 11                      | 95                  | 95                     | 0                      | 4                       | 3.06                 | 0                | 95.96            |
| Low dose group| 11                      | 114                 | 114                    | 0                      | 5                       | 3.39                 | 0                | 95.80            |
| Medium dose   | 10                      | 98                  | 98                     | 0                      | 2                       | 2.00                 | 0                | 98.00            |
| High dose group| 11                     | 119                 | 119                    | 2                      | 3                       | 2.42                 | 1.65             | 95.97            |

Note: fetus live rate (%) = number of live fetus / numbers of implantation

Note: if there is No * marked on the shoulder of the same column of data in the table, it means that there is no significant difference when compared with the control group ($P>0.05$).
Table 4. Effects of the feeding L-tryptophan on the growth of fetal rats

| Dose group     | Number of pregnant rats | Litter rat / nest | Weight (g) | Body length (cm) | Tail length (cm) | Placenta weight (g / litter) |
|----------------|-------------------------|-------------------|------------|------------------|------------------|-----------------------------|
| Normal control | 11                      | 8.6±1.9           | 3.94±0.32  | 3.63±0.17        | 1.28±0.09        | 0.73±0.10                   |
| Low dose group | 11                      | 8.9±1.2           | 3.71±0.37  | 3.56±0.18        | 1.25±0.09        | 0.66±0.14                   |
| Medium dose group | 10                   | 9.8±1.4           | 3.8±0.45    | 3.49±0.21        | 1.21±0.08        | 0.66±0.12                   |
| High dose group | 11                      | 9.7±1.1           | 4.12±0.32   | 3.67±0.16        | 1.30±0.09        | 0.65±0.08                   |

Note: if there is No * marked on the shoulder of the same column of data in the table, it means that there is no significant difference when compared with the control group (P>0.05).

3.5. Effect on fetal rat deformity

There is no significant difference (P>0.05) between the test group fed with the feeding L-tryptophan and the normal control group in the appearance deformity rate, skeleton deformity and internal organ deformity, such as malformation fetus rate, live fetus malformation rate and maternal malformation fetus rate (Table 5).

Table 5. Teratogenic effect of the feeding L-tryptophan on rats

| Dose group     | Number of pregnant rats | Number of live litters | Number of malformation fetus | Number of maternal malformation fetus | Live fetus malformation average rate (%) | Maternal malformation fetus rate (%) | Appearance | Skeleton | Internal organs |
|----------------|-------------------------|------------------------|-------------------------------|---------------------------------------|------------------------------------------|-------------------------------------|------------|----------|-----------------|
| Normal control | 11                      | 95                     | 1                             | 1                                     | 1.05                                     | 9.09                                | 0          | 1        | 0               |
| Low dose group | 11                      | 114                    | 1                             | 1                                     | 0.88                                     | 9.09                                | 0          | 1        | 0               |
| Medium dose group | 10                   | 98                     | 0                             | 0                                     | 0                                        | 0                                   | 0          | 0        | 0               |
| High dose group | 11                      | 119                    | 1                             | 1                                     | 0.84                                     | 9.09                                | 0          | 1        | 0               |

Note: if there is No * marked on the shoulder of the same column of data in the table, it means that there is no significant difference when compared with the control group (P>0.05).

4. Discussion

In the study of teratogenesis, it can be divided into traditional teratogenesis test and feeding reproduction teratogenesis test. It is mainly due to the different time to give the test substance. The traditional teratogenesis test is to give the test substance within the time of organ formation during embryo development, and the feeding reproduction teratogenesis test is divided into first-generation, second-generation or third-generation feeding reproduction test. In the first generation feeding reproduction test, the test substance is given during the formation of sperm or egg until the fetus is delivered, and then investigate the teratogenicity of the test substance. In the second or third generation reproduction teratogenesis test, we continue to give the test substance after the weaning of the offspring until the F2 or F3 generation is delivered, and investigate the teratogenicity to the offspring after a long time of contact of the test substance.

In teratogenesis, the most sensitive stage to teratogens is the organogenetic period, which is generally called the critical or dangerous stage. The traditional teratogenesis test is to give the test
substance in the organogenetic period to let us understand the teratogenesis of the test substance to the embryo. In common experimental animals, the organogenetic period of rats, calculated from the date of fertilization, is about 9-17 days, small mice is 7.5-16 days, and rabbits is 11-20 days. In organogenetic period, the contact between teratogen and embryo may cause abnormal morphology and structure. However, if the contact between teratogen and embryo occurs in the blastocyst formation stage before implantation, embryo often dies and there are few deformities. The blastocyst formation period of rats, calculated from the date of fertilization before implantation, is about 3-4 days, and the time of implantation beginning is about 5.5-6 days; small mice is 3-4 days and 4.5-5 days; rabbits is 3-4 days and 7 days [9].

L-tryptophan is one of the three amino acid feed additives except tyrosine and methionine. If the L-tryptophan supply in animal body is insufficient, there will be a series of maladjustment symptoms in animals, such as slow accumulation of fat and slow growth of the body. In male animals, testicular dysplasia sometimes occurs [10]. With the continuous improvement of fermentation technology, L-tryptophan, as a feed additive, its application prospect will be more and more broad.

5. Conclusion
In the acute toxicity experiment, the oral LD50 of rats fed with the feeding L-tryptophan was more than 20.0g/kg b.w. According to the WHO acute toxicity classification standard, the non-toxic level is that the LD50 of rats is more than 15.0g/kg b.w., which indicates that L-tryptophan is non-toxic. The traditional teratogenicity test of the feeding L-tryptophan for the test substance is negative, which shows that it has no obvious teratogenesis effect on rats, and even in the number of corpus luteum, the number of implantation, the number of live litter and other aspects, L-tryptophan is significantly better than the normal control, indicating that L-tryptophan is safe in teratogenesis when used as feed additive.

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