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Acute and sub-acute oral toxicological evaluations and mutagenicity of *N*-carbamylglutamate (NCG)

Xin Wu [a, b], Dan Wan [a, *], Chunyan Xie [a], Tiejun Li [a], Ruilin Huang [a], Xugang Shu [b, c], Zheng Ruan [b], Zeyuan Deng [b], Yulong Yin [a, b, **]

[a] *Observation and Experiment Station of Animal Nutrition and Feed Science in South-Central China, Ministry of Agriculture, Hunan Provincial Engineering Research Center for Healthy Livestock and Poultry Production, Key Laboratory of Agro-ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, Chinese Academy of Sciences, Changsha 410125, China*

[b] *State Key Laboratory of Food Science and Technology and College of Life Science and Food Engineering, Nanchang University, Nanchang 330031, China*

[c] *Guangzhou Tanke BIO-TECH Co. Ltd., Guangzhou 510800, China*

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ABSTRACT

N-carbamylglutamate (NCG) is a metabolically stable analog of *N*-acetylglutamate that activates carbamyl phosphate synthase-1, a key arginine synthesis enzyme in enterocytes. It is a promising feed additive in swine in China. In this study, we assessed the acute and sub-acute toxicity of NCG in Sprague–Dawley (SD) rats. All rats survived until they were killed at a scheduled time point. No adverse effects or mortality was observed following acute oral administration of 5000 mg/kg NCG to SD rats. No biologically significant or test substance-related differences were observed in body weights, feed consumption, clinical signs, a functional observational battery, organ weights, histopathology, ophthalmology, hematology, coagulation, and clinical chemistry parameters in any of the treatment groups in sub-acute doses of NCG at target concentrations corresponding to 500, 2000, and 3000 mg/kg/day for 28 days neither. In addition, no evidence of mutagenicity or genotoxicity was found, either *in vitro* in bacterial reverse mutation assay or *in vivo* in mice bone marrow micronucleus assay and sperm shape abnormality assay. On the basis of our findings, we conclude that NCG is a non-toxic substance with no genotoxicity.

1. Introduction

N-acetylglutamate (NAG) (Fig. 1A) is an endogenous essential cofactor. It activates a key enzyme (carbamylphosphate synthetase-1, CPS1) of the arginine-synthetic pathway (Wu et al., 2004). It is used clinically in the treatment of N-acetylglutamate synthase deficiency, organic acidurias, and maple syrup urine disease (Bachman et al., 1982; Ucar et al., 2009; Schwahn et al., 2010).

N-carbamylglutamate (NCG) (Fig. 1B), a structural analog of NAG, has been shown to stimulate citrulline and arginine synthesis in enterocytes (Wu et al., 2004) and increase endogenous synthesis of arginine, plasma concentrations of arginine and somatotropin, growth rate, and muscle protein synthesis in sow-reared piglets (Wu et al., 2004; Frank et al., 2006, 2007). Furthermore, dietary NCG supplementation can increase intestinal growth and heat shock protein-70 expression in weanling pigs (Wu et al., 2010). Nowadays, it is becoming a promising feed additive in many countries, especially in China.

In vitro and *in vivo* evidence showed that NAG is not genotoxic or acutely toxic, with a no-observed-adverse-effect-level (NOAEL) for systemic toxicity from sub-acute (28-day) dietary exposure to NAG was 914 mg/kg of body weight/day for male rats and 1007 mg/kg of body weight/day for female rats (Harper et al., 2009). No mortalities or evidence of adverse effects was observed in SD rats following acute oral gavage with NAG at a dose of 2000 mg/kg BW or 100, 500, or 1000 mg/kg BW/day for 28 days. However, limited information was found in NCG toxicity.

Considering its promising application prospect of the NCG as a feed additive, its toxicity investigation needs to be performed

Abbreviations: NCG, *N*-carbamylglutamate; NAG, *N*-acetylglutamate; CPS1, carbamyl phosphate synthetase 1; Arg, arginine; NAGS, NAG synthase; GH, growth hormone; SD, Sprague–Dawley; BMM, bone marrow micronucleus; LD_{50}, median lethal dose; MPCEs, microscope for the presence of micronuclei; PCEs, polychromatic erythrocytes; LC/MSD, mass spectrometry detection; HPLC, high-performance liquid chromatography; ANOVA, one-way analysis of variance.

* Corresponding author.
** Corresponding author.
E-mail addresses: w.dan@isa.ac.cn (D. Wan), yinyulong@isa.ac.cn (Y. Yin).

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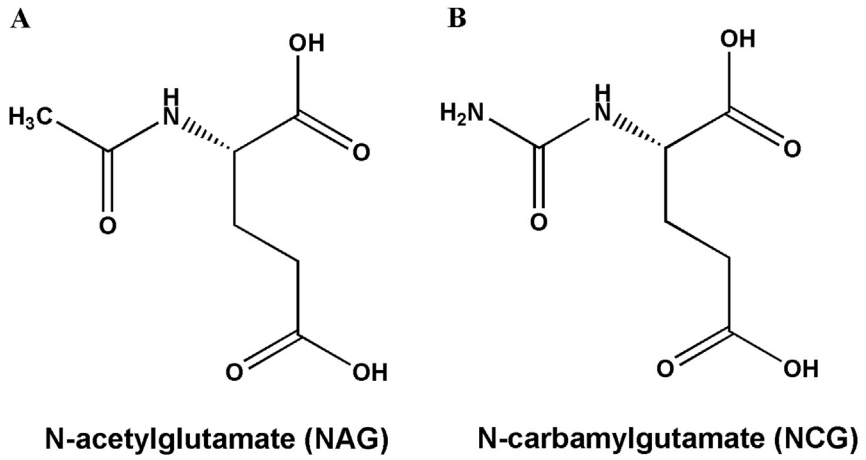

Fig. 1. Chemical structures of *N*-acetylglutamate (NAG) (A) and *N*-carbamylglutamate (NCG) (B).

urgently. Therefore, the present study was conduct to assess the potential genotoxicity and acute and sub-acute (28-day) oral toxicity of NAG. All studies here were conducted in accordance with Good Laboratory Practice guidelines (OECD, 1995, 1997; 2001; FDA, 2010).

2. Materials and methods

2.1. Materials

NCG ($C_6H_{10}N_2O_5$, CAS 1188-38-1, MW 190.15 g/mol, purity 98.30%, lot number Z20110301) was produced by L&K (Hunan) technology Co., LTD (Changsha, China). 4-Nitroquinoline N-oxide ($C_9H_6N_2O_3$, CAS 56-57-5), cyclophosphamide (CP, $C_7H_{17}Cl_2N_2O_3P$, CAS 50-18-0) were purchased from Aladdin Reagent Co., LTD (Shanghai, China). Phenobarbital/benzoflavone (10%)-induced rat liver S9 was purchased from Platt Bio-Pharmaceutical Co., Ltd. (Beijing, China). New-born calf serum was produced by Hangzhou Sijiqing Biological Materials Limited Company (Hangzhou, China). The rat and mice diet were procured by the Comparative Medicine Centre of Yangzhou University (Yangzhou, China). All other reagents used were of analytical grade or better, as indicated in the certificate of analysis supplied by the manufacturer.

2.2. Animals and treatment

Specific pathogen-free (SPF) Sprague–Dawley (SD) rats (8 weeks, 180–220 g) and Kunming mice (6 weeks, 18–22 g) were purchased from the Comparative Medicine Centre of Yangzhou University (Yangzhou, China). The basic feed without any drugs was produced according to the Chinese standard "Laboratory animal rats and mice feed" (GB14924.3, 2010). Animals were kept in a barrier-maintained animal room conditioned at a temperature of 25 ± 3 °C, a relative humidity of 50 ± 10% and a 12-h light/dark cycle. They were housed 4 or 5 per cage with hardwood shavings as bedding, and received basic feed and fresh water freely during the experiment period. All animals had a one-week acclimatization period before experiment started. Use of animals was in accordance with "Guide for the Care and Use of Laboratory Animals" NIH Publication (NRC, 1996). All the animal studies were approved by the Ethical Committee of the Faculty of Veterinary Medicine at Yangzhou University.

2.3. Acute oral toxicity study

SD rats (10 males and 10 females) and Kunming mice (10 males and 10 females) used in this study were obtained from Comparative Medicine Centre of Yangzhou University (license No.: SCXK (Su) 2007–0001). Feed was withdrawn overnight before administration of starting doses at 12 h. An acute, one-day oral toxicity study was conducted in accordance with the Organization for Economic Co-operation and Development (OECD) Guideline 423 (OECD, 2001). As our preliminary test revealed a relatively low toxicity of NCG in rats and mice, a dose of 5000 mg/kg NCG dissolved in water was administered to male and female SD rats via oral gavage. Control groups were administered water equally. Then rats and mice that survived were observed for another 14 days. Observations included evaluation of skin and fur, eyes and mucous membranes, respiratory, autonomic effects (e.g. salivation), central nervous system effects (tremors and convulsions), changes in the level of motor activity, gait and posture, reactivity to handling and stereotypes or bizarre behavior (e.g. self-mutilation, walking backwards). The time of death was recorded as precisely as possible. At the end of the test, surviving animals were sacrificed. All studies were conducted in accordance with FDA Good Laboratory Practice guidelines (FDA, 2010).

2.4. Sub-acute toxicity study

2.4.1. Experimental design

Male and female SD rats (8 weeks old, 219.39 ± 8.56 g) were obtained from Comparative Medicine Centre of Yangzhou University (license No.: SCXK (Su) 2008–0004), and randomly assigned to four groups (0, 500, 2000, or 3000 mg/kg/day, 28 days) by a computer-generated (weight-ordered) randomization procedure. Each group consisted of 10 female rats and 10 male rats. Dietary NCG concentrations were adjusted weekly according to the average male body weights for each dosage group from the previous week and the historical mean feed consumption values (approximately 30 g/rat/day), to maintain consistent daily (mg/kg) doses. All rats were anesthetized with pentobarbital sodium and killed at the 28th day after an overnight fast. This study followed the OECD Guideline No.407 (OECD, 1995) and was conducted in compliance with FDA Good Laboratory Practice Regulations (FDA, 2010). The endpoints included clinical and ophthalmological observations, body weight and feed consumption values, a functional observational battery, motor activity, hematology and coagulation assessment, serum chemistry levels, and clinical pathology.