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Establishment of health food safety evaluation method-I*n vivo* genotoxicity evaluation by micronucleus assay

Yi-Wei Lai [1, #], Pi-Hsin Chen [2, #], Ya-Ling Cyue [2], Ya-Peng Wang [2], Yu-Ying Fang [2], Shih-Yi Guo [2], Chia-Ying Lin [2], Keng-Chia Hsu [2], Tsung-Han Wu [2], Yan-Zhong Wu [2], Yu-Hsing Lin [1] and Shao-Wen Hung [2, 3, *]

[1] *Department of Pet Healthcare, Yuanpei University of Medical Technology, Xiangshan, Hsinchu 300, Taiwan.*
[2] *Division of Animal Industry, Animal Technology Research Center, Agricultural Technology Research Institute, Xiangshan, Hsinchu 300, Taiwan.*
[3] *Department of Nursing, Yuanpei University of Medical Technology, Xiangshan, Hsinchu 300, Taiwan.*
[#] *Contributed equally to this work.*

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Abstract

In recent years, various factors such as shifts in health consciousness, changes in lifestyle habits, increased focus on dietary intake, the promotion of alternative medical concepts, and advancements in life sciences and technology have propelled the robust growth of the health food industry. An increasing number of reports suggest that "functional foods," containing beneficial functional components, may enhance short-term well-being and are increasingly regarded as healthful. Building upon these findings, this study aims to enhance the production capacity of high-value agricultural products. However, for quality assurance, toxicity assessment of functional foods is imperative. There is a growing necessity to employ *in vivo* genotoxicity assays to evaluate the carcinogenic potential of these products. In this study, we tried to establish another *in vivo* genotoxicity evaluation platform via acridine orange induction. According to all results in this study, during the experiment, there were no statistically significant differences in BW between the negative control group and the normal control group ($p > 0.05$). Moreover, the clinical observations of the experimental mice showed that all mice survived until the end of the experiment, and no abnormal clinical symptoms were observed. The mouse's food consumption in each group were monitored during the experiments. The food consumption of mice was recorded daily after the experiment. During the experiments, there were no significant difference between two groups on the food consumption. The percentage of RETs/1,000 RBCs in the negative control group was significantly decrease than the normal control group ($p < 0.001$). RETs/1,000 RBCs (‰) in ICR mice (n = 5/group) in the negative control group and the normal control group at 48th and 72th hours-experiment were respectively 18 ± 4.6 / 45.6 ± 4.0 (48th hours-experiment) and 19 ± 4.8 / 47.6 ± 4.8 (72th hours-experiment). The percentage of Mn-RETs/1,000 RETs in the negative control group was significantly increase than the normal control group ($p < 0.001$). Mn-RETs/1,000 RETs (‰) in ICR mice (n = 5/group) in the negative control group and the normal control group were 10.6 ± 2.1 / 0.4 ± 0.5 and 8.2 ± 1.8 / 0.4 ± 0.5, respectively. Taken all results of RET/RBCs (‰) and MN-RET/RETs (‰) together, the genotoxicity in the negative control group has been successfully induced. In light of these requirements, we propose utilizing the mammalian erythrocyte micronucleus assay to detect damage induced by test substances to erythroblast chromosomes. This study endeavors to establish a sensitive and stable method for quantifying micronuclei formation in erythrocytes *in vivo*. The resulting data will facilitate the evaluation of potential toxicity associated with agricultural functional products in the future.

Keywords: Genotoxicity; ICR Mice; *In vivo*; Micronucleus assay; Reticulocytes

* Corresponding author: Shao-Wen Hung

1. Introduction

In recent years, various factors such as shifts in health consciousness, changes in lifestyle habits, increased focus on dietary intake, the promotion of alternative medical concepts, and advancements in life sciences and technology have propelled the robust growth of the health food industry. With the world's population gradually aging and a rising market demand in Association of Southeast Asian Nations (ASEAN) countries, the functional food market is expected to flourish [1-6].

Research and investigations have indicated that Taiwan's agricultural science papers are cited more frequently than those in other fields, showcasing Taiwan's significant research and development prowess in agricultural science and technology. The recent focus on the industrialization of scientific and technological achievements has garnered attention from both the government and the public. This global trend presents an opportunity for Taiwan's agricultural science and technology to once again demonstrate its innovative capabilities [7-13].

While considerable resources and technologies have been accumulated in upstream research and development, the challenge lies in swiftly transforming these technologies and resources into marketable commodities through industrialization platforms, thereby fostering settlements and industries. Consequently, there is an urgent need to establish a stable, accurate, and rapid animal test evaluation system to assess the safety of health foods and functional foods as a pre-market screening tool [14-19].

An increasing number of reports suggest that "functional foods," containing beneficial functional components, may enhance short-term well-being and are increasingly regarded as healthful. Building upon these findings, this study aims to enhance the production capacity of high-value agricultural products. However, for quality assurance, toxicity assessment of functional foods is imperative. There is a growing necessity to employ *in vivo* genotoxicity assays to evaluate the carcinogenic potential of these products [20-23].

In light of these requirements, we propose utilizing the mammalian erythrocyte micronucleus assay to detect damage induced by test substances to erythroblast chromosomes. This study endeavors to establish a sensitive and stable method for quantifying micronuclei formation in erythrocytes *in vivo*. The resulting data will facilitate the evaluation of potential toxicity associated with agricultural functional products in the future.

2. Materials and Methods

2.1. Chemicals and Reagents

Cyclophosphamide monohydrate (CPP; Sigma-Aldrich, Cat. No. C0768), acridine orange solution (Sigma-Aldrich, Cat. No. 65-61-2), reverse osmosis water, phosphate-buffered saline (PBS; Sigma-Aldrich, Cat. No.P3813), saline (Taiwan Biotech Co., LTD, Cat. No. 100-120-1101), and Zoletil 50 (Virbac, Carros, France) were used in this study.

2.2. Experimental Animals and Experimental Design

Adult male ICR mice [6 weeks old; 10 mice; body weight (BW) between 26-27g)] with specific pathogen-free conditions were used for this study, were purchased from BioLASCO Taiwan Co., Ltd. (Yilan, Taiwan). These ICR mice were fed with standard laboratory diet (No. 5001, LabDiet®; PMI Nutrition International, St. Louis, MO, USA) and distilled water ad libitum during the experimental period. The environment was maintained room temperature (24-27°C) and 60%-70% humidity with a photoperiod of 12-hr light/12-hr dark cycle. The study will begin after a week acclimation. The Institutional Animal Care and Use Committee (IACUC) of Agricultural Technology Research Institute inspected all animal experiments and this study comply with the guidelines of protocol IACUC-112016 approved by the IACUC ethics committee. The 10 ICR male mice were divided respectively into as the normal control group (n = 5) and the negative control group (n = 5). The CPP-induced mice' genotoxicity via intraperitoneal injection were performed in the negative control group. In the normal control group, the mice were treated with reverse osmosis water, via oral administration. The clinical behaviors, BW, food consumption, and blood smear exanimation were monitored and performed during the experiment.

2.3. Collection of Peripheral Blood from ICR Mice

The ICR mice were anesthetized with Zoletil 50 and the blood collection site was cleaned with 70% alcohol. The blood was stored in anticoagulant tubes containing K_2-EDTA for use in the subsequent experiments.

2.4. Preparation of Blood Smear and Acridine Orange Staining with Fluorescence Microscopic Examination

After 48 and 72 hours post-administration of the test substance, blood samples were collected via performing submandibular blood collection from the restrained mice, and 5 μL of blood was obtained to prepare blood smears. Blood was placed on pre-dyed slides with 0.1% acridine orange solution, gently covered with a cover slip, and spread evenly to a single layer thickness of blood cells. After incubating at room temperature and avoiding light for 3 hours, the number of reticulocytes and micronucleated reticulocytes in the blood were observed under a fluorescence microscope. The 1,000 reticulocytes were observed in each ICR mouse, the number of micronuclei was recorded, and the proportion of reticulocytes in total red blood cells was calculated.

2.5. Statistical Analysis

The data of BW, reticulocyte percentage, and reticulocyte micronucleus incidence rate for each group of mice were expressed as mean and standard deviation (SD). Mice' BW were analyzed using one-way ANOVA followed by Duncan's multiple range test in SPSS statistical software. Reticulocyte percentage and micronucleated reticulocyte incidence rate were analyzed using SPSS software to calculate the median and Mann–Whitney U test. The $p < 0.05$ indicates significant differences between groups.

3. Results

3.1. Normality of Mice' Clinical Behavior in Two Groups

In this study, the clinical behavior observation indexes of mice in each group were normal during the experiments. During the experiments, the mice in each group had smooth hair, normal hair color, and the normal activity. Moreover, all mice were survival until the end of the experiments. The survival rate was 100% (10/10) (Table 1).

Table 1 Mice' clinical observation daily in each group during the experiment

Group	Administration	Clinical observation daily			
		Day 1	Day 2	Day 3	Day 4
Normal control	Reverse osmosis water	N	N	N	N
Negative control	50 mg/kg body weight CPP	N	N	N	N

N: normal

3.2. Change of Mice' BW in Two Groups

The BW of mice was detected on day 1 and day 4. During the experiment, the mice' BW continued to rise and there was no statistically significant difference in BW between 2 groups (Table 2).

Table 2 The change of mice' body weight in each group during the experiment

Group	Administration	Body weight (g)	
		Day 1	Day 4
Normal control	Reverse osmosis water	33.8 ± 1.4	34.0 ± 1.6
Negative control	50 mg/kg body weight CPP	34.9 ± 0.8	35.8 ± 1.1

All data are expressed as mean ± SD. CPP: Cyclophosphamide monohydrate. Each group was 5 ICR mice (n = 5/group).

3.3. No Significant Change of Mouse's Food Consumption in All Groups

In this study, the mouse's food consumption in each group were monitored during the experiments. The food consumption of mice was recorded daily after the experiment. During the experiments, there were no significant difference between two groups on the food consumption (data not shown).

3.4. Change of RETs/1,000 RBCs and Mn-RETs/1,000 RETs Percentages during the Experiment

Mice' peripheral anticoagulant blood were collected at 48th and 72th hours-experiment, respectively. Blood samples were prepared for blood smears and processed with acridine orange staining. After blood sample processing, they were

examined by using fluorescence microscope. The percentages of RETs/1,000 RBCs and Mn-RETs/1,000 RETs were evaluated. The results showed that ICR mice in the negative control group were induced genotoxicity. The percentage of RETs/1,000 RBCs in the negative control group was significantly decrease than the normal control group ($p < 0.001$) (Figure 1A-B). RETs/1,000 RBCs (‰) in ICR mice (n = 5/group) in the negative control group and the normal control group at 48th and 72th hours-experiment were respectively 18 ± 4.6 / 45.6 ± 4.0 (48th hours-experiment) and 19 ± 4.8 / 47.6 ± 4.8 (72th hours-experiment) (Figure 1A-B). The percentage of Mn-RETs/1,000 RETs in the negative control group was significantly increase than the normal control group ($p < 0.001$) (Figure 1C-D). Mn-RETs/1,000 RETs (‰) in ICR mice (n = 5/group) in the negative control group and the normal control group were 10.6 ± 2.1 / 0.4 ± 0.5 and 8.2 ± 1.8 / 0.4 ± 0.5, respectively (Figure 1C-D). Taken all results of RET/RBCs (‰) and Mn-RET/RETs (‰) together, the genotoxicity in the negative control group has been successfully induced.

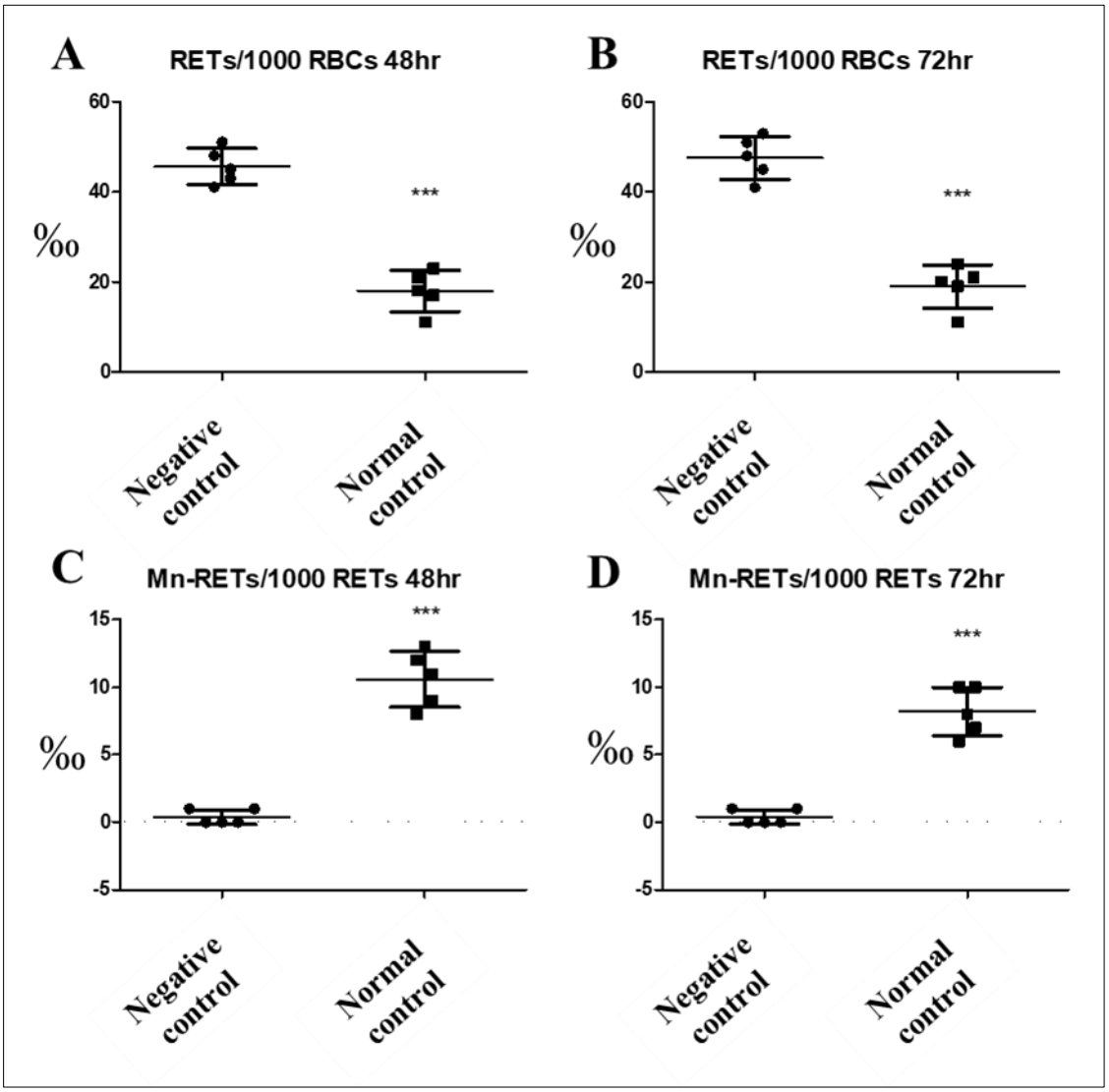

Figure 1 Change of RET/RBCs and Mn-RET/RETs in each group at 48th hour- and 72th hour-experiment. (A) RETs/1,000 RBCs (‰) at 48th hours-experiment. (B) RETs/1,000 RBCs (‰) at 72th hours-experiment. (C) Mn-RETs/1,000 RETs (‰) at 48th hours-experiment. (D) Mn-RETs/1,000 RETs (‰) at 72th hours-experiment. All data are expressed as mean ± SD. All significant differences compared to negative control group were reported at $^{***}p < 0.001$.

4. Discussion

In order to evaluate the safety of health food, Taiwan government has formulated a method for evaluating the safety of health food [28]. Among health food safety assessment methods, genotoxicity test methods can be divided into *in vivo* and *in vitro* tests, the purpose of which is to detect the genetic damage and extent directly or indirectly caused by the test substances, including the microbial gene mutation assays, the *in vitro* genotoxicity analysis of mammalian cells, and the genotoxicity analysis of animals *in vivo* [24-26]. *In vivo* genotoxicity assays in animals generally were used chromosomal damage assays of rodent hematopoietic cells, including bone marrow cell micronucleus assays, chromosomal abnormalities assays or peripheral blood micronucleus assays. Due to the high sensitivity of the micronucleus assays, this method is the most commonly used method for testing toxic drug-induced chromosomal aberrations *in vivo* [27-28].

Our previous results have been published for establishing the genotoxicity evaluation platform via mitomycin C induction [28-30]. During the in vivo genotoxicity-evaluated experiment, the experimental animal's clinical behavior, BW, food consumption, and the percentage of RETs/RBCs (reticulocytes/red blood cells) and Mn-RETs/RETs (micronucleated reticulocytes/reticulocytes) were evaluated. Both sexes ICR mice were given three daily treatments by intraperitoneal injection of 2 mg/kg of mitomycin C (genotoxicity induction) or by oral route of 200 μL of PBS (normal control group). Until 48h after the last treatment, K_2-EDTA-anticoagulated peripheral blood specimens were collected. These blood samples were processed for the microscopy-based analysis using Giemsa stain and the percentage of reticulocytes and micronucleated reticulocytes was determined. The results were shown that the experimental mice' clinical behaviors were normal in all groups. The BW and food consumption were no significant difference between all groups. RETs/RBCs (‰) in male or female ICR mice in the negative control group and the normal control group were respectively 7.8 ± 0.8 / 8.6 ± 0.8 and 23.2 ± 1.5 / 22.1 ± 1.3; Mn-RETs/RETs (‰) in male or female ICR mice in the negative control group and the normal control group were 2.0 ± 0.0 / 2.0 ± 0.0 and 43.2 ± 10.6 / 39.6 ± 10.9, respectively. Both RETs/RBCs (‰) and Mn-RETs/RETs (‰) in male or female ICR mice in the negative control group were significantly difference than the normal control group ($p < 0.001$) [28-30].

In this study, we tried to establish another *in vivo* genotoxicity evaluation platform via acridine orange induction. According to all results in this study, during the experiment, there were no statistically significant differences in BW between the negative control group and the normal control group ($p > 0.05$). Moreover, the clinical observations of the experimental mice showed that all mice survived until the end of the experiment, and no abnormal clinical symptoms were observed. The results of reticulocyte count in CPP-treated ICR mice at 48 hours showed that the median reticulocyte percentage in the normal control group was 45‰, while in the negative control group it was 18.0‰. The reticulocyte percentage in the negative control group was significantly lower than that in the normal control group. The results of micronucleated reticulocyte count in CPP-treated ICR mice at 48 hours showed that the median micronucleus incidence rate in the normal control group was 0‰, while in the negative control group it was 11‰. The micronucleus incidence rate in the negative control group was significantly higher than that in the normal control group. The results of reticulocyte count in CPP-treated ICR mice at 48 hours showed that the median reticulocyte percentage in the normal control group was 48‰, while in the negative control group it was 20‰. The reticulocyte percentage in the negative control group was significantly lower than that in the normal control group. The results of micronucleated reticulocyte count in CPP-treated ICR mice at 72 hours showed that the median micronucleus incidence rate in the normal control group was 0‰, while in the negative control group it was 8‰. The micronucleus incidence rate in the negative control group was significantly higher than that in the normal control group. Comparison of mitomycin C-induced *in vivo* genotoxicity in ICR mice and acridine orange-induced *in vivo* genotoxicity in ICR mice revealed that the RETs/RBCs (‰) and Mn-RETs/RETs (‰) in the negative control group were all significantly different from those in the normal control group [28-30].

5. Conclusion

The purpose of this study was to establish in vivo genotoxicity evaluation platform via inducing ICR mice' genotoxicity with CPP. After the experiment, all groups of experimental mice showed normal BW gain, with no abnormal clinical symptoms or mortality observed. Observation of CPP-treated ICR mice at 48 and 72 hours revealed that the reticulocyte percentage in the negative control group was significantly lower than that in the normal control group, while the micronucleus incidence rate was significantly higher than that in the normal control group. Taken these results together, we successfully established the phenomenon of micronucleus in peripheral blood cells induced by CPP in ICR mice. In the future, we hope this genotoxicity mouse platform will provide to detect the genotoxicity in the test samples.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest.

Statement of ethical approval

The Institutional Animal Care and Use Committee (IACUC) of Agricultural Technology Research Institute inspected all animal experiments and this study comply with the guidelines of protocol IACUC 112016 approved by the IACUC ethics committee.

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