Cytotoxicity evaluation of the whole protein extract from *Bar*-transgenic rice on *Mus musculus* lymphocytes

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Genet. Mol. Res. 16 (1): gmr16019331
Received September 21, 2016
Accepted September 21, 2016
Published January 23, 2017
DOI http://dx.doi.org/10.4238/gmr16019331

ABSTRACT. With the expanding demand for genetically modified (GM) rice, its safety evaluation is of great significance. Therefore, this study was carried out to assess the acute cytotoxicity of the whole protein extract from GM rice *Bar*68-1 in *Mus musculus* lymphocytes *in vitro*. Cell viability was determined by Cell Counting Kit-8 (CCK-8) and Neutral Red Uptake (NRU) tests. CCK-8 tests was carried out according to the manufacturer's instructions. Cell dehydrogenase (catalytic redox enzymes) activity was spectrophotometrically determined at 450 nm. The tests result were recorded immediately. NRU tests were completed under yellow light in a dark room according to an improved protocol. Lysosomal uptake of neutral red was spectrophotometrically determined at 540 nm and the results were recorded immediately. The results showed that the survival rate of *M. musculus* lymphocytes in the positive control group was significantly less than in the blank control group ($P < 0.05$). Moreover, an exposure- time-effect relationship

was observed in the positive control group with CCK-8 and NRU tests. There was no significant difference in survival rate between GM rice *Bar*68-1group and non-GM rice D68 group ($P > 0.05$). The GM rice *Bar*68-1 group also did not show a higher survival rate than non-GM rice D68 group ($P > 0.05$). These results suggested that the whole protein extract from *Bar*68-1 and D68 were equivalent in their cytotoxicity, and GM rice *Bar*68-1 had no acute cytotoxic effect on *M. musculus* lymphocytes *in vitro*.

Key words: *Bar*-transgenic rice; *Mus musculus*; Cytotoxicity; *In vitro*; Survival rate

INTRODUCTION

Rice is a major food crop in China and the world, so its production is of great significance for providing basic nutrition and food safety. Different from traditional breeding methods, transgenic technology can transfer a gene fragment of an organism to another genome, breaking the species reproductive isolation barrier, to change genetic traits and get the desired attribute, which can realize the communication of genetic material between animal, plant, and microorganism. In order to prevent exposure to genes harmful to the environment, animals, and human health, and control biosafety risk from being transferred to receptor biology in the process of operation, governments around the world have attached great importance to the safety and assessment of genetically modified (GM) organisms.

To this day, it is unclear whether transgenic rice is harmful to human health, or toxic to cells, tissues, and organs. It has become an increasingly controversial issue, and has caused widespread concern worldwide in recent years (Gu, 2005; Jiao and Li, 2007; Key et al., 2008; Zhang et al., 2009). With the development of transgenic crop areas and the expanding domestic demand for GM food, its safety evaluation is of great significance (Millstone et al., 1999; Pereira, 2000; Liu et al., 2009; Gong et al., 2012).

Globally substantial research evaluating the safety of transgenic rice has been done. These studies have been mainly in the discipline of toxicology (animal experiments) involving analysis of metabolites (Anldanl et al., 2002; Zhao et al., 2013; Jiang et al., 2014), toxicokinetics (Kuiper et al., 2004; Delaney et al., 2008), chronic toxicity and sub-chronic toxicity (Kuiper et al., 1999; Hug, 2008), and reproductive and teratogenicity test methods (Conner and Jacobs, 1999; König et al., 2004; Feng et al., 2013; Wang et al., 2014). Safety evaluations were made, mainly by feeding the animal model, to detect the potential effect of transgenic rice on animal organs (König et al., 2004; Gu, 2005; Kok et al., 2008; Fu et al., 2012). The whole test process is time-consuming, has a heavy workload, and a high cost. Conventional methods of toxicology test tissue and organ pathological changes without reference to the cellular changes. In order to make an objective evaluation of GM rice cytotoxicity, this study has used the Cell Counting Kit-8 (CCK-8) and Neutral Red Uptake (NRU) tests to detect the degree of cellular damage caused by the whole protein extract from transgenic rice on *M. musculus* lymphocytes in a shorter time, with a lighter workload, and a low cost.

MATERIAL AND METHODS

Materials

Lymphocytes and rice used

M. musculus (Kunming mice) lymphocytes, the target cells, were provided by the heart development laboratory of College of Life Science, Hunan Normal University.

Bar-transgenic rice *Bar*68-1 [Production License: Agriculture Basic Security Examination (2006) No. 060] and the corresponding non-transgenic rice D68 were purchased from the Institute of Subtropical Agricultural (ISA), Chinese Academy of Science.

Extraction of rice protein

Fresh 10 g *Bar*68-1 or D68 rice was mixed with liquid nitrogen and ground to a powder in a mortar, and then dissolved in Western and immunoprecipitation (IP) cell lysate (BiYunTian Biotechnology Research Institute) (Shanghai, China) and mixed thoroughly. Phenylmethylsulfonyl fluoride (PMSF) was added to the lysates, a few minutes before use, such that the PMSF final concentration was 1 mM. A 50-mg rice powder sample was taken and 400 μL of Western and IP cell lysate was added (adding 100-200 μL lysate per 20 mg of sample) until they fully cracked, then centrifuged for 3-5 min at 10000-14000 *g* to get the supernatant, which was then filtered using the Microcon Ultra-0.5 3000 super filters (Millipore Company, USA) in 1.5 mL centrifuge tubes at 14,000 *g*. After the filtrate was discarded, 450 μL of cooled (4°C) 1X phosphate buffered saline (PBS) was added to the supernatant and centrifuged at 14,000 *g*, then the pellet in the centrifuge tube was discarded. This step was repeated again. The centrifuge tube was replaced by a new 1.5-mL tube and the ultrafiltration device inverted and centrifuged at 1000 *g* to collect the rice protein. The extracted rice protein was dissolved in PBS using ultrasonic oscillation. Finally, the concentration of the rice protein was determined by a bicinchoninic acid (BCA) protein assay kit, and standardized to 1 mg/mL. The solution was kept at -80°C.

Experimental design

One mL *M. musculus* lymphocyte suspension (at a density of 50,000 cells/mL) was seeded into a 24-well culture plate to culture cells at 37°C. The exposure concentration of the whole protein extract from *Bar*68-1 rice and D68 rice was set as 25, 50, 100, and 200 μg / mL, and the exposure time for each concentration was 2, 6, and 24 h. PBS and vincristine (Amresco, USA) were administered to the blank control group and the positive control group, respectively. All the experiments were repeated three times.

Cell viability assay

Cell viability was determined by CCK-8 and NRU tests. CCK-8 tests were carried out

according to the manufacturer's protocol (Dojindo, Japan) and also as described by Jiang et al. (2004). Cell dehydrogenase (catalytic redox enzymes) activity was determined at 450 nm using a spectrophotometer (Tecan, France). The test result was recorded immediately. NRU tests were completed under yellow light in a dark room according to the improved steps as described by Putnam et al. (2002) and Canal- Raffin et al. (2008). Lysosomal uptake of neutral red was determined as described by Geh et al. (2006) at 540 nm using a spectrophotometer (Tecan, France), and the results were recorded immediately.

Statistical analysis

All data are reported as the means ± standard deviation. The data were subjected to analysis of variance (ANOVA) using the SPSS 17.0 statistical software. If the variance was homogeneous, the data were compared using the least significant difference (LSD) method; else, if the variance was not homogeneous, Dunnett's T3 was used. $P < 0.05$ was considered to indicate statistical significance.

RESULTS

Effect of the whole protein extract from GM rice *Bar*68-1 on cell viability determined by CCK-8 tests

As shown in Table 1 and Figure 1, the CCK-8 tests showed that over a period of time the survival rate of the positive control group was lower than that of the blank control group, and the difference was significant ($P < 0.05$). After lymphocytes were incubated with vincristine (0.025 μg/mL) for 2, 6, and 24 h, their survival was 79.50, 73.99, and 59.58%, respectively. It was clear that there was a certain relationship between cellular damage and incubation time. There was no significant difference in survival rate between the GM rice *Bar*68-1 group and non-GM rice D68 group ($P > 0.05$). However, compared with the blank control group, the survival rate of GM rice *Bar*68-1 group was lower, but there was no significant difference between them ($P > 0.05$).

Table 1. The survival rate of lymphocytes (means ± SD; %) detected by the CCK-8 tests *in vitro*.

Treatment	Concentration (μg/mL)	Time		
		2 h	6 h	24 h
Blank control	-	100	100	100
Positive control	0.025	79.50 ± 1.20*	73.99 ± 0.95*	59.58 ± 1.12*
The whole protein of GM rice *Bar*68-1	25.0	93.84 ± 3.57	92.04 ± 3.73	93.42 ± 4.12
	50.0	93.91 ± 4.41	91.71 ± 3.44	94.21 ± 3.24
	100.0	91.30 ± 3.24	93.90 ± 4.71	93.46 ± 4.43
	200.0	94.11 ± 3.50	92.04 ± 3.96	92.43 ± 2.87
The whole protein of non-GM rice D68	25.0	93.56 ± 4.11	94.31 ± 3.92	93.88 ± 4.25
	50.0	92.45 ± 3.33	93.96 ± 2.85	94.57 ± 3.66
	100.0	94.06 ± 4.40	92.52 ± 4.04	93.94 ± 3.87
	200.0	91.42 ± 3.55	92.81 ± 4.22	92.85 ± 4.35

*Compared with blank control group, $P < 0.05$.

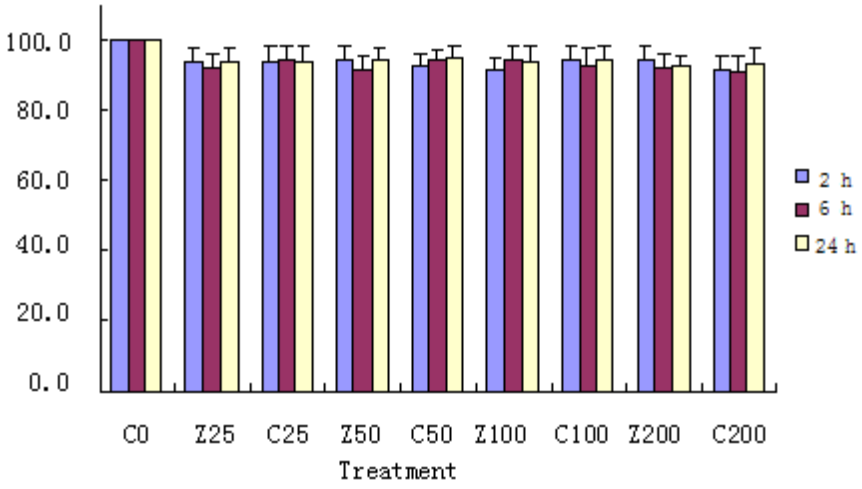

Figure 1. Comparison of survival rate of lymphocytes detected by the CCK-8 tests. Note: C0 represents blank control; Z25, Z50, Z100, and Z200 represent 25, 50, 100, and 200 μg/mL of the whole protein extract from GM rice, respectively; and C25, C50, C100, and C200 represent 25, 50, 100, and 200 μg/mL of the whole protein extract from non-GM rice, respectively.

Effect of the whole protein extract from GM rice *Bar*68-1 on cell viability determined by NRU tests

As shown in Table 2 and Figure 2, the NRU tests showed that over a period of time the survival rate of the positive control group was lower than that of the blank control group, and the difference was significant ($P < 0.05$). After lymphocytes were incubated with vincristine (0.025 μg/mL) for 2, 6, and 24 h, their survival rate was 81.31, 74.90, and 60.55%, respectively. It became obvious that there was a certain relationship between cellular damage and incubation time.

There was no significant difference in survival rate between GM rice *Bar*68-1 group and non-GM rice D68 group ($P > 0.05$). However, compared with the blank control group, the survival rate of GM rice *Bar*68-1 group was lower, but there was no significant difference between them ($P > 0.05$).

Table 2. The survival rate of lymphocytes (means ± SD; %) detected by neutral red uptake (NRU) tests *in vitro*.

Treatment	Concentration (μg/mL)	Time		
		2 h	6 h	24 h
Blank control	-	100	100	100
Positive control	0.025	81.31 ± 1.35	74.90 ± 1.16*	60.55 ± 1.28
The whole proteins of GM rice *Bar*68-1	25.0	92.78 ± 4.52	93.93 ± 3.26	91.39 ± 3.14
	50.0	94.85 ± 3.23	93.64 ± 4.21	92.12 ± 4.26
	100.0	93.24 ± 5.32	94.85 ± 3.89	92.35 ± 3.45
	200.0	92.74 ± 4.12	91.95 ± 4.58	93.36 ± 4.24
The whole protein of non-GM rice D68	25.0	93.55 ± 3.58	92.85 ± 2.95	91.78 ± 3.57
	50.0	92.44 ± 5.42	91.86 ± 3.68	93.15 ± 5.63
	100.0	94.07 ± 4.24	95.43 ± 4.35	92.84 ± 4.82
	200.0	91.44 ± 3.31	93.73 ± 5.11	94.23 ± 3.94

*Compared with blank control group, $P < 0.05$.

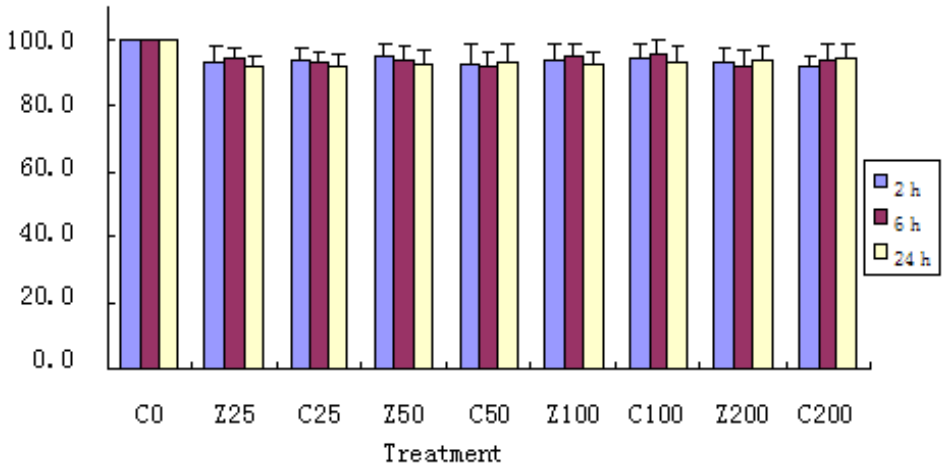

Figure 2. Comparison of survival rate of lymphocytes detected by the NRU tests. Note:C0 represents the blank control; Z25, Z50, Z100, and Z200 represent 25, 50, 100, and 200 μg/mL of the whole protein extract from GM rice, respectively; and C25, C50, C100, and C200 represent 25, 50, 100, and 200 μg/mL of the whole protein extract from non-GM rice, respectively.

DISCUSSION

Both CCK-8 and NRU tests are cytotoxicity tests, but the detection mechanism of each is different. The CCK-8 tests detects the survival rate of cells by evaluating cellular dehydrogenase activity while the NRU test measures the lysosomal uptake of a dye. A combination of these two tests can effectively improve the sensitivity and accuracy of cytotoxicity detection. Therefore, in this study, these tests with different detection mechanisms for cytotoxicity were selected. According to the toxicological test requirements, the exposure time was set to 2, 6, and 24 h, so that observations were made of lymphocytes survival rate in the short term to investigate the exact relationship between cytotoxicity and exposure time.

Based on CCK-8 and NRU tests, we found that there was a significant difference in the survival rate of lymphocytes between the blank control group and the positive control group in 3 different incubation periods ($P < 0.05$). It was observed that the survival rate of lymphocytes indicated an obvious cellular damage-incubation time relationship, and the results proved that CCK-8 and NRU tests are sensitive and effective in determining lymphocyte death induced by vincristine. The results suggested that there was no significant difference in survival rate between GM rice *Bar*68-1 group and non-GM rice D68 group ($P > 0.05$). Similarly, the difference in the survival rate of lymphocytes between GM rice *Bar*68-1 group and blank control group was not significant ($P > 0.05$). Hence, the study showed that the whole protein extract from GM rice *Bar*68-1 and non-GM rice D68 had equivalent cytotoxic effect. Furthermore, GM rice *Bar*68-1 had no acute cytotoxic effect on *M. musculus* lymphocytes *in vitro*, in concordance with results obtained with transgenic rice Bt63 that did not show significant cytotoxicity in human lymphocytes, as described by Chen et al. (2012).

In the acute toxicity tests, lymphocytes were incubated for 2, 6, and 24 h with multiple doses of the whole protein extract from GM rice *Bar*68-1 to determine the short-term(in a shorter time) cytotoxicity and characteristics of the whole protein extract. Therefore, the test

results only proved that the whole protein extract of GM rice *Bar*68-1 had no cytotoxicity in short-term tests. As for the question whether whole protein extracts of GM rice *Bar*68-1 have sub-chronic or chronic cytotoxicity, further studies are needed.

The whole protein extract from GM rice *Bar*68-1 and non-GM rice D68 are equivalent in cytotoxic effect. GM rice *Bar*68-1 has no acute cytotoxic effect on *M. musculus* lymphocytes *in vitro* due to the following reasons: 1) The expression product of the foreign gene *bar* is phosphinthricin acetyltransferase (PAT), which is present in *Bar*-transgenic herbicide resistant rice. PAT acetylates the free amino of phosphinothricin (PPT), which is the main component of glufosinate in herbicides. However, PAT cannot restrain the activity of glutamine synthetase. Therefore, PAT can induce transgenic crops to become resistant to the herbicide, and the toxicity caused by glufosinate is eliminated. Thus, PAT has no acute cytotoxicity in the organisms' cells. 2) The amount of PAT expressed in plants is too low to cause cytotoxic effect on *M. musculus* lymphocytes. Thus, PAT is relatively safe (Liu et al., 2007).

ACKNOWLEDGMENTS

Research supported by a project grant from National Natural Science Foundation of China (Grant #31172107).

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