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RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

A 22 week subchronic feeding study of transgenic BADH alfalfa in rabbits

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Abstract

With the increasing use of genetically modified organisms (GMO) worldwide, the controversy of their safety would be unlikely to abate. Salt is a major stress limiting crop productivity and most alfalfa are salt-sensitive. In plants expression of the gene encoding for Betaine Aldehyde Dehydrogenase (BADH) confers tolerance to salinity and drought stresses. The safety of genetically modified alfalfa was assessed in rabbits consuming diets containing transgenic alfalfa (50%) and conventional non-genetically modified alfalfa (50%). Twenty four male rabbits were fed the two types of alfalfa for 22 weeks. Feed intake and weight gain were measured. Haematology and blood biochemistry of the two groups were compared at both 11th and the 22nd week. All the animals were sacrificed at the end of the experiment and the parameters of the organ index, gross and microscopic appearance of tissues were compared between the two groups. No adverse effects were observed in rabbits consuming GM alfalfa and non-GM alfalfa. The results indicated that the feeding value of GM alfalfa and conventional non-GM alfalfa were equal in this study. No detrimental expected effects were observed in rabbits fed genetically modified alfalfa. We concluded that it is as safe and nutritious as existing alfalfa.

Keywords: BADH-transgenic Alfalfa, safety assessment, substantial equivalence, rabbits

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Introduction

Alfalfa (*Medicago sativa* L.) is the most important forage crop in many parts of the world. It is recognized for its capacity to produce large yields of highly nutritious palatable feed and for its excellent soil-improving ability (Li et al., 2010). Salt stress is a major stress limiting crop productivity and most alfalfa are salt-sensitive (Jin et al., 2010). In order to protect themselves from these adverse conditions, most organisms have developed certain defence mechanisms. One such mechanism is the accumulation of osmoprotectants like betaines. Betaines are amino acid derivatives with a fully methylated nitrogen atom. They carry no net charge at physiological pH and are nontoxic even at high concentrations (Livingstone et al., 2003). Glycine betaine is synthesized by a two-step oxidation of choline (Choline is first oxidized to betaine aldehyde–catalyzed by a ferredoxin-dependent choline monooxygenase and the betaine aldehyde is then oxidized to glycine betaine mediated by an NAD-dependent BADH (Burnet et al., 1995; Rathinasa-bapathi et al., 1997). BADH activity is induced by osmotic stress condition (Rhodes Hanson, 1993).

Plants have the gene encoding for BADH to enhance tolerance to salinity and drought stresses (Kumar et al., 2004). The advent of molecular genetic technology allows us to address these issues much more efficiently than in the past. Generating new germplasm of alfalfa that has stress resistance is one of the basic ways to use new lands for cultivation and improve crop productivity.

BADH gene was introduced into alfalfa (Medicago sativa L.) plants by *Agrobacterium tumefaciens* mediated transformation. Using the T0 generation of transgenic plants with BADH gene as testing material, alfalfa was bred with breeding objectives such as growing behaviour, flower colour, biomass and stress resistance and so on. After a few generations of breeding, alfalfa with a new germ plasm for salt tolerant (Winicov and Krishnan, 1996). The current feeding

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study was conducted for the purpose of safety assessment to determine the safely of genetically modified alfalfa.

Materials and Methods

Test and control substances

Alfalfa containing BADH gene and its non-transgenic near isogenic control alfalfa were grown under controlled conditions at isolated fields. Test plots within a controlled field test site were separated by bare ground and/or temporal isolation. Test plots and containers holding the harvested grain were labelled and the harvesting equipment was cleaned between harvesting of each test plot.

Animal care and management

Twenty-four male rabbits, approximately one month old and weighing between 450~750g were selected for this study. The experimental and control groups of rabbits fed twice at 8:00 in the morning and 16:00 at evening. All rabbits were provided with food and tap water *ad libitum*. Rabbits were housed individually in stainless steel, wire-mesh cages suspended above cage boards. Cage and rack location within the animal room was rotated every week.

Study design and diet administration

Following 3 days of acclimatization, rabbits were randomly distributed equally by body weight into two experimental groups so that there was no apparent difference between the body weights of the two groups. Fresh diet was supplied weekly. Rabbits in the experimental group were fed diets formulated with 50% (wt/wt) BADH alfalfa, while those in the control group were fed diets formulated with the same concentrations of near-isogenic control alfalfa. Animals were fasted overnight (approximately 15 h) but did have access to water prior to blood collection.

Clinical observations

All animals were observed twice daily for mortality and once daily for abnormal behaviour and/or appearance. Physical examination was performed weekly. Detailed weekly clinical observations in a standardized arena were recorded by exception and included evaluation of coat condition, skin, eyes, mucous membranes, occurrence of secretions and excretions, autonomic nervous system activity (lacrimation, piloerection, unusual respiratory pattern), changes in gait, posture, response to handling, and presence of clonic, tonic, stereotypical or bizarre behaviour. Animals were tested for 22 weeks.

Haematology

At the end of the experiment, the blood was collected from ear margin vein in two batches. One sample was used with sodium citrate as an anticoagulant for the determination of white blood cell (WBC), red blood cell (RBC), haemoglobin (Hb) and hematocrit (HCT). These parameters were measured with the help of PE-6800 Fully Auto Haematology Analyzer (Shenzhen, China).

Serum chemistry

Second batch of blood samples were centrifuged to separate serum for determination of albumin (ALB), total protein (TP), triglycerides (TG), blood urea nitrogen (BUN), alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose (GLUC) which were performed with 7020 Automatic Biochemical Analyzer (Hitachi, Japan).

Anatomic pathology

Rabbits were fasted for at least 15 h, at the end of the feeding experiment and gross necropsy of major organs was conducted by visual inspection. Following euthanasia, the following organs were excised and weighed: heart, liver, spleen, lung, kidneys, small intestine, large intestine, stomach, thymus and testis. The relative weight of each organ (or paired organ weights) was determined based on final individual body weights. Tissue sections from heart, liver, spleen, lung, kidneys and intestine were fixed with buffered formalin and embedded in paraffin and stained with hematoxylin and eosin. Histopathological examination of tissue sections was conducted at the Experimental Animal Research Centre, Shandong Agricultural University (Taian, China).

Statistical analysis

Data from groups of rabbits consuming diets formulated with alfalfa containing BADH gene were compared to its non-transgenic near isogenic control group. Homogeneity variance was analyzed by one-way analysis of variance (ANOVA). Analysis was performed using Statistical Product and Service Solutions (SPSS) v18.0 (SPSS Inc., Chicago, IL, USA). Baseline data were presented as MEAN ± SD. Results with a value of $P<0.05$, two-tailed were considered statistically significant.

Results

Effects of transgenic or non-transgenic alfalfa diet on the haematology of rabbits

Results of the group feeding BADH alfalfa and the control groups at study interim and termination are shown in Fig. 1. There was no significant difference between these two groups in all the 4 haematological parameters at all the 3 periods (W_0, W_{11} and W_{22}) of the study ($P>0.05$).

Effects of transgenic or non-transgenic alfalfa diet on the biochemical parameters in rabbits

Biochemical analyses showed no significant differences among the rabbits in these two groups ($P>0.05$). Results of blood chemical analyses are shown in Fig. 2 (from A to G). The ALT and AST concentration

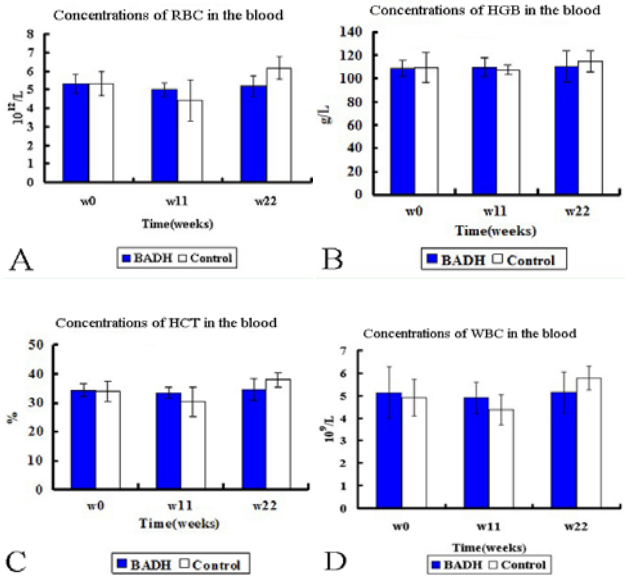

Fig. 1 (A): Value of RBC in rabbits of two groups following 22 weeks of exposure to transgenic or non-transgenic alfalfa in the diet, (B): Value of Hb in rabbits of two groups following 22 weeks of exposure to transgenic or non-transgenic alfalfa in the diet, (C): Value of HCT in rabbits of two groups following 22 weeks of exposure to transgenic or non-transgenic alfalfa in the diet, (D):Value of WBC in rabbits of two groups following 22 weeks of exposure to transgenic or non-transgenic alfalfa in the diet. W0: Day zero; W11, W22: at the end of 11 and 22 weeks treatment.

of transgenic group was slightly higher than that of non-transgenic group at the end of 11 and 22 weeks, which is consistent with the parameters at the start (W_0) of the experiment. No apparent differences in the other blood biochemical composition between rabbits fed transgenic or non-transgenic alfalfa in the diet were observed.

Effects of transgenic or non-transgenic alfalfa diet on weight and daily feed intake

The weight and daily feed intake of the two groups at the end of treatment (W22) are shown in Table 1. There was no significant difference ($P>0.05$) between the feed intake and body weight of the two groups.

Effects of transgenic or non-transgenic alfalfa diet on the weight of the organs

The organ weight to body weight ratios of the two groups at the end of the treatment (W_{22}), are shown in Table 2. There were not significant difference ($P>0.05$) on the weight of the main organ (heart, liver, spleen, lung, kidneys, small intestine, large intestine, stomach, thymus and testis) of rabbits.

Gross and microscopic anatomic pathology

The histological photomicrographs of the heart, liver, spleen, lung, kidneys, and large intestine sections are

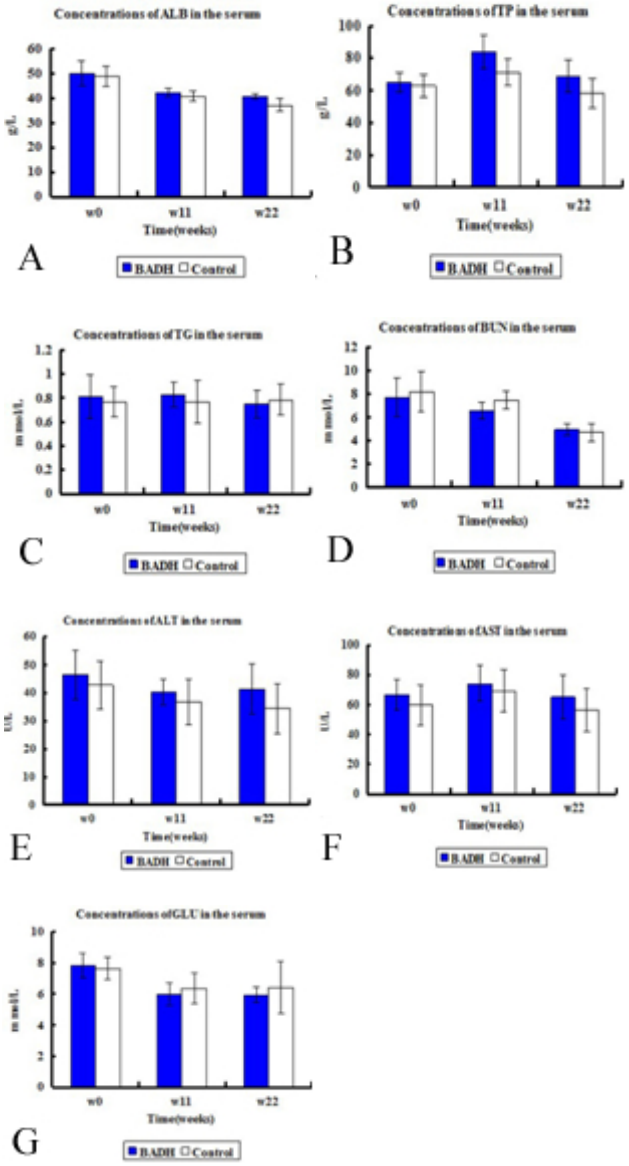

Fig. 2: (A): Value of ALB in rabbits of two groups following 22 weeks of exposure to transgenic or non-transgenic alfalfa in the diet, (B): Value of TP in rabbits of two groups following 22 weeks of exposure to transgenic or non-transgenic alfalfa in the diet, (C): Value of TG in rabbits of two groups following 22 weeks of exposure to transgenic or non-transgenic alfalfa in the diet, (D):Value of BUN in rabbits of two groups following 22 weeks of exposure to transgenic or non-transgenic alfalfa in the diet, (E): Value of ALT in rabbits of two groups following 22 weeks of exposure to transgenic or non-transgenic alfalfa in the diet. (F): Value of AST in rabbits of two groups following 22 weeks of exposure to transgenic or non-transgenic alfalfa in the diet. (G): Value of GLU in rabbits of two groups following 22 weeks of exposure to transgenic or non-transgenic alfalfa in the diet. W0: basal time, W11, W22: at the end of 11, 22 weeks treatment.

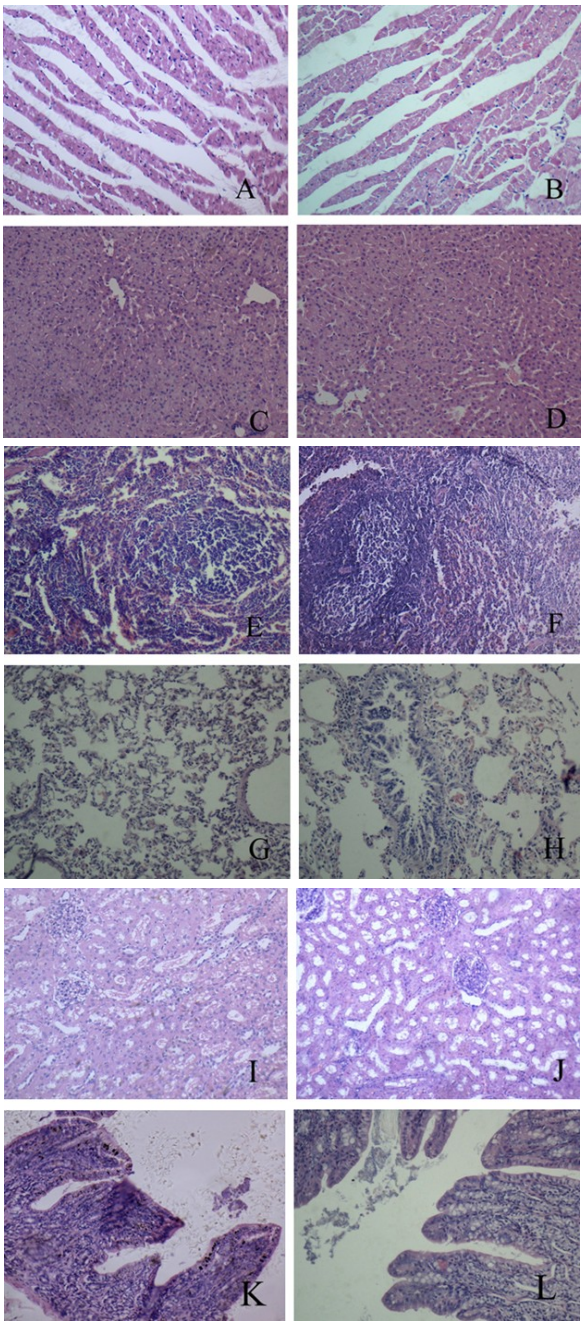

Fig. 3: Histopathology of the organs in rabbits exposed to transgenic or non-transgenic alfalfa for 22 weeks. Sections were stained with Haemtoxylin and Eosin.

Light microscopy showing the heart tissue (×200) of experiment group as shown in (A), normal heart tissue (×200) as shown in (B), the liver tissue (×200) of experiment group as shown in (C), normal liver tissue (×200) as shown in (D), the spleen tissue (×200) of experiment group as shown in (E), normal spleen tissue (×200) as shown in (F), the lung tissue (×200) of experiment group as shown in (G), normal lung tissue (×200) as shown in (H), the kidney tissue (×200) of experiment group as shown in (I), normal kidney tissue (×200) as shown in (J), the large intestine tissue (×200) of experiment group as shown in (K), normal large intestine tissue (×200) as shown in (L).

Table 1: Body weight and daily feed intake in control and treated rabbits

Group	Alfalfa containing BADH gene	near-isogenic control alfalfa
Original weight (kg)	0.61±0.05	0.63±0.05
11th week weight (kg)	1.83±0.09	1.95±0.04
22nd week weight (kg)	2.95±0.04	3.06±0.07
Daily intake (g/d)	110.43±1.20	113.66±1.30

Table 2: Organ/body weight ratios

Parameter	BADH	Control
Heart	0.227±0.005	0.283±0.053
Liver	5.279±0.128	4.633±0.276
Spleen	0.035±0.002	0.045±0.008
Lung	0.367±0.057	0.487±0.061
Kidneys	0.537±0.003	0.588±0.109
Small intestine	3.687±0.123	3.003±0.445
Large intestine	6.702±0.261	5.871±1.430
Stomach	1.487±0.053	1.252±0.227
Thymus	0.329±0.012	0.313±0.052
Testis	0.166±0.021	0.168±0.019

shown in Fig. 3. The array and cytoarchitecture of the cardiomyocytes, nephrocyte, hepatocyte and enterocyte were normal and complete among all the groups. There was no demolition and disappearance of the splenic corpuscles. The structure of red pulp, white pulp and splenic sinusoid were normal and clear. No evidence of increased incidence of pathologic changes was observed in the organs and tissues of rabbits in the experimental group compared with rabbits in the control group.

Discussion

The exogenous gene might bring in acute toxicity and allergenic proteins or some unintended pleiotropic changes in application, which could affect hematopoietic system and inflammation markers. Furthermore, inflammatory cytokines adversely affect erythropoiesis as well as RBC life span (Weiss et al., 2005). Severe reductions in blood elements (RBC, HGB and HCT) may lead to anemia, wide interferences with oxygen transport to tissue and may induce hypoxia, which in turns will further promote the anemia process (Mona et al., 2011). For this reason, the concentration of RBC, Hb and HCT are the most important parameters reflecting anaemia and iron deficiency (Carlo et al., 2011).

AST and ALT activities provide a sensitive and specific measure of hepatic function or injury (Abbès et al., 2006). Under pathological conditions including cirrhosis, adverse effects of some drugs or foods, these enzymes will be leaked into the plasma, thus raising their activity, so they are biomarkers of the hepatocytes (Nyblom et al., 2004). The changes of the TG and GLU, as an important site responsible for the glucose and lipid metabolism also suggest alterations in the hepatic injury and function.

Albumin and globulin constitutes the total plasma proteins (TPP) and are in ratio 1:2. Thus, albumin

constitutes the major component of the TPP. It has a half life of 120 days and its level is lowered in chronic liver disease such as cirrhosis and in poor diet or states of impaired protein catabolism (Nwangwu et al., 2011). Blood urea nitrogen (BUN) is commonly used to detect kidney toxicity in preclinical and clinical studies as well as in routine clinical care (Bonventre et al., 2010).

Our results show that all the haematological and serum chemistry data of the two groups were not significantly different ($P>0.05$) at both week 11 and week 22, which were confirmed and supported by the relative organ weights of spleens, liver and kidney and their histological findings. The weight and daily intake were also not significantly different ($P>0.05$). As spleen is important immunological organs and its organ indexes may to some extent reflect the strength of the immune function (Xiao et al., 2011). The results might imply that exogenous gene did not exert a direct damage on the hematopoietic, biochemical and immune system functions of rabbits.

As the genes encoding BADH proteins were originally isolated from *Atriplex hortensis* and the materials used in genetic engineering (pBin-438 vector, 35S promoter, NOS Terminator, marker gene NPTII and report gene GUS) have no history of being harmful to animals and humans, the proteins expressed in genetically modified BADH Alfalfa is safe to animals and humans.

It is always complex in practice to give an assessment of GM crops using feeding studies. On one hand, it could make the animals be malnutrition if we try to feed them the whole food exclusively in the diet for a lone time, meanwhile, some crop contains anti-nutritional factors itself like protein lectin and all of these can result in the generation of un-interpretable data (Fengyun et al., 2012).

On the other hand, many uncertain factors will happen in the process of compound feed development like the degradation of DNA and the other rough material may also contain related genetically modified ingredients which can easily lead to the pollution of gene. So it is necessary to formulate specialized and nutritionally balanced diets for different laboratory animals in different experiments. Only in this way can we make sure the animals are in normal physiological condition and the data we get from the experiments are meaningful (Hui et al., 2005).

The feeding value of GM alfalfa and conventional non-GM alfalfa were equal in this study, and no detrimental effects were observed in rabbits fed alfalfa genetically modified with BADH gene, confirming that it is as safe and nutritious as existing alfalfa.

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