

Mice fed on a diet enriched with genetically engineered multivitamin corn show no sub-acute toxic effects and no sub-chronic toxicity

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Received 30 April 2012;

revised 5 July 2012;

accepted 10 July 2012.

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Summary

Multivitamin corn is a novel genetically engineered variety that simultaneously produces high levels of β -carotene, ascorbate and folate, and therefore has the potential to address simultaneously multiple micronutrient deficiencies caused by the lack of vitamins A, B9 and C in developing country populations. As part of the development process for genetically engineered crops and following European Food Safety Authority (EFSA) recommendations, multivitamin corn must be tested in whole food/feed sub-chronic animal feeding studies to ensure there are no adverse effects, and potential allergens must be identified. We carried out a 28-day toxicity assessment in mice, which showed no short-term sub-acute evidence of diet-related adverse health effects and no difference in clinical markers (food consumption, body weight, organ/tissue weight, haematological and biochemical blood parameters and histopathology) compared to mice fed on a control diet. A subsequent 90-day sub-chronic feeding study again showed no indications of toxicity compared to mice fed on control diets. Our data confirm that diets enriched with multivitamin corn have no adverse effects on mice, do not induce any clinical signs of toxicity and do not contain known allergens.

Keywords: genetically engineered corn, safety assessment, sub-chronic toxicity.

Introduction

Up to 50% of the world's population suffers from multiple micronutrient deficiencies reflecting an over-dependence on foods that lack essential vitamins and minerals. The best way to address such deficiencies is to diversify the diet, particularly by including fresh fruits and vegetables. However, this is impractical in many developing country settings where the population lacks access to diverse foods and subsists on a predominantly cereal-based diet (Farre *et al.* 2010a, 2011a). Strategies to overcome micronutrient deficiencies include the provision of supplements, the fortification of processed food such as flour and salt, the implementation of breeding programs to generate nutrient-rich varieties of staple crops and the use of genetic engineering to develop staple foods with higher micronutrient contents (Gomez-Galera *et al.*, 2010). Strategies that tackle micronutrient deficiency at source by creating nutrient-rich crops are known as biofortification approaches (Farre *et al.*, 2011b). These should be targeted at staple crops such as rice and corn, which are the primary source of calories for more than 75% of the human population (Food and Agriculture Organization, 2009).

Genetic engineering is the most versatile approach for biofortification because it is not limited by the diversity available within the gene pool of the staple crop and can be implemented directly in local elite cultivars without complex breeding programs (Farre *et al.*, 2011b). Staple crops can therefore be engineered to produce much higher nutrient levels than

can be achieved by conventional breeding, and breeding lines can be generated much more rapidly. There have been many reports of genetically engineered staple crops accumulating high levels of vitamins (reviewed in Zhu *et al.*, 2007; Farre *et al.*, 2010b; Bai *et al.*, 2011). Most of these enhanced varieties are still at the laboratory testing phase or in early field trials, but at least one is very near to broad release. This is Golden Rice that is engineered with two genes conferring the ability to synthesize β -carotene (pro-vitamin A) in the endosperm (Ye *et al.*, 2000; Paine *et al.*, 2005). The advantage of multivitamin corn is that it addresses multiple nutrient deficiencies simultaneously and as such it represents a 'next generation' nutritional intervention.

There are two potential solutions to this challenge, the first to develop nutritionally enhanced staples that provide such high levels of a key target nutrient that a very small portion offers the full dietary reference intake. Mixed meals of different enhanced staples could then provide a full nutritional complement. This is unlikely to be practical because it would rely on the simultaneous availability of many varieties as well as user compliance to ensure the correct consumption ratio to achieve nutritional completeness while avoiding nutrient toxicity. The second solution is to develop staple crops simultaneously enhanced with different nutrients, providing a balanced and nutritionally complete meal in a manageable portion. Such crops would be all but impossible to generate by conventional breeding even if sufficient genetic diversity were available, because many different traits would need to

be targeted simultaneously (Naqvi *et al.*, 2010). We have begun this process by developing a prototype multivitamin corn simultaneously engineered to accumulate high levels of β -carotene, ascorbate and folate, therefore addressing deficiencies for vitamins A, B9 and C (Zhu *et al.*, 2008; Naqvi *et al.*, 2009). Transgenic lines were created by introducing four genes representing three different metabolic pathways into the elite South African white corn inbred M37W and selecting those with the highest nutrient content. The best performing line produced 57 $\mu\text{g/g}$ dry weight β -carotene (>169-fold increase), 106.94 $\mu\text{g/g}$ dry weight ascorbate (>six-fold increase) and 200 $\mu\text{g/g}$ dry weight folate (twofold increase) in the kernels (Naqvi *et al.*, 2009). These are biologically relevant levels, taking into account that 100–200 g of multivitamin grain provides the full RDI of β -carotene (provitamin A), an adequate intake of folate and 20% of RDI in the case of ascorbate.

Genetic engineering provides the only practical approach to develop nutritionally complete staples but one limitation of genetically engineered crops is the onerous regulatory environment (Ramessar *et al.*, 2010; Sabalza *et al.*, 2011). Such crops must undergo extensive tests that are not required of conventional varieties even if they are genetically identical, because the trigger for testing is the process used to generate the crops not the actual product (in Europe and many developing countries). The assessment of nutritionally enhanced varieties created by genetic engineering must include (among other tests) compositional analysis, laboratory feeding trials in animals to test sub-chronic toxicity as well as tests for allergenicity and nutritional assessments (Kuiper *et al.*, 2001; König *et al.*, 2004).

The aim of 28-day toxicity assessment is to identify immediate or short-term toxic effects, whereas that of sub-chronic toxicity evaluations is to determine any adverse effects caused by repeated exposure over a longer period. These assessments involve young animals and are designed to reveal signs of toxicity in organs, tissues and cells over a period long enough for major toxic effects to become apparent without any age-associated change in tissue morphology or function (van Haver *et al.*, 2008). In the case of genetically engineered plants, the evaluations are based on substantial equivalence or comparative assessment with conventional varieties of the same crop.

As part of the development of our multivitamin corn, we therefore compared the proximates composition of multivitamin corn and nontransgenic corn diets and we carried out a 28-day toxicity feeding study in mice, revealing no short-term sub-acute evidence of diet-related adverse health effects in male or female mice, and no differences between the tested animals and those fed with control diets in terms of food consumption, body weight, organ/tissue weight, haematological and biochemical blood parameters and histopathology. We then carried out a 90-day sub-chronic feeding study, again with no indications of toxicity compared to mice fed on control diets. Our data confirm that diets enriched with multivitamin corn are not toxic in animals and do not induce clinical abnormalities. The confirmed safety of multivitamin corn in animal tests therefore opens the way for its use in human populations. This is the last step on the long road to market authorization which should make multivitamin corn available to impoverished and malnourished populations in developing countries.

Results

Compositional analysis

There were minimal differences in proximate levels between the multivitamin corn and nontransgenic corn, which did not have any impact on nutrition (supporting information Table S1).

Twenty-eight-day sub-acute toxicity assessment

The animals in all three groups exhibited normal clinical signs before and during the assessment. There were no signs of abnormal appearance or behaviour, no unusual droppings and no aversion to any of the diets. No mice died during the experiment.

There was no statistically significant difference ($P < 0.05$) in either food consumption or body weight among the three diet groups when comparing the whole groups or individual sexes. After 28 days, the male body weight was 23.88 ± 1.82 g in the reference diet group, 25.24 ± 0.47 g in the nontransgenic corn group and 24.81 ± 0.29 g in the multivitamin corn group. The corresponding female body weights were 20.28 ± 1.33 , 19.13 ± 1.32 and 20.69 ± 0.74 g, respectively. There were no statistically significant differences ($P < 0.05$) between the multivitamin corn group and either of the controls in terms of haematological parameters, biochemical markers, organ weights or histopathology.

Ninety-day sub-chronic toxicity assessment

The animals in all three groups exhibited normal clinical signs before and during the assessment. There were no signs of abnormal appearance or behaviour, no unusual droppings and no aversion to any of the diets. No mice died during the experiment.

After 13 weeks, there was no statistically significant difference ($P < 0.05$) in either food consumption (Figure 1) or body weight (Figure 2) among the three diet groups when comparing the whole groups or individual sexes, although males fed on the multivitamin corn diet were on average marginally heavier (27.76 ± 0.75 g) than their counterparts in the other two groups (27.23 ± 1.01 g for the reference diet, 27.04 ± 1.13 g for the wild type corn diet). The females in the reference, wild type and multivitamin diet groups weighed 21.94 ± 1.44 g, 22.39 ± 1.42 g and 22.24 ± 0.99 g, respectively.

There were no statistically significant differences ($P < 0.05$) between the multivitamin corn group and either of the controls in terms of biochemical markers (Tables 1 and 2) or haematological parameters (Tables 3 and 4).

Blood samples were analysed to determine the effect of each diet on known disease markers and to identify correlations between organ weights and histopathological findings. Because only a small amount of blood was available, we focused on a subset of the tests: ALT, alanine transaminase normally present in the liver and heart, which is used to evaluate the health of those organs; BUN, blood urea nitrogen, which is a marker of kidney disease; total cholesterol, which is a cardiovascular disease risk biomarker; total proteins, which is a marker of immune system dysfunction as well as liver and kidney complications; and albumin, as an indicator of liver damage and nutritional status.

We assessed standard haematological values such as the white blood cell count (WBC) and the differential white blood cell count (neutrophils, lymphocytes, monocytes, eosinophils

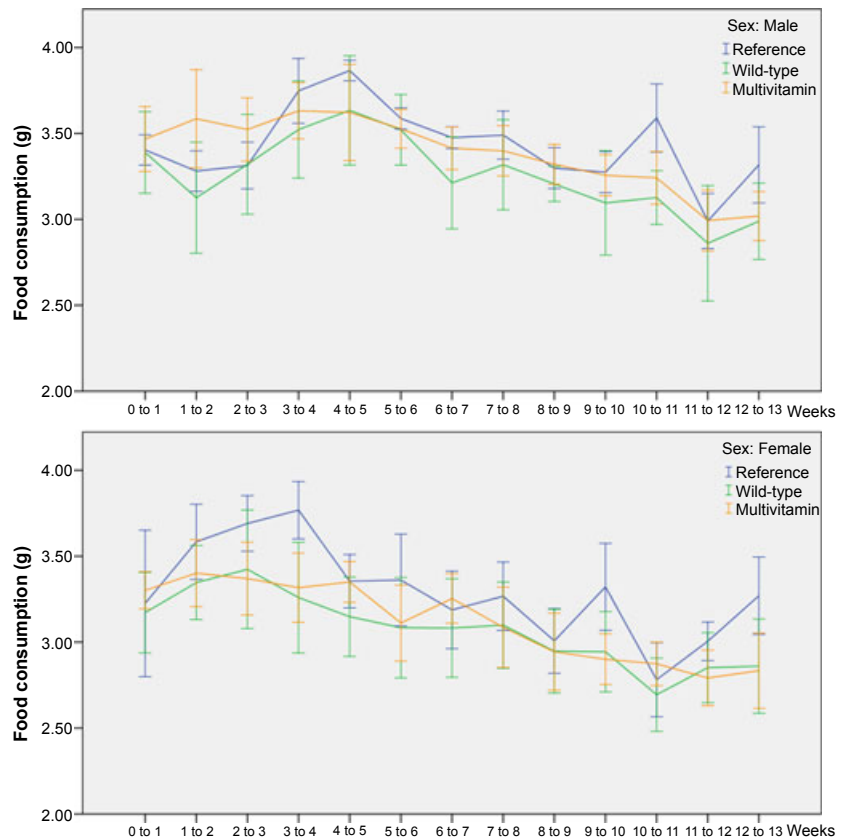


Figure 1 Mean weekly feed consumption (mean $\pm$ CI 95%) of male and female mice. Animals were fed rodent diet (reference group), and rodent diets containing wild type and multivitamin corn for 13 weeks.

and basophils). We also assessed the red blood cell count (RCB), haemoglobin content (HGB), haematocrit (HTC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentration (MCHC) to identify potential preliminary immunotoxicity or anaemia caused by liver disease, folate deficiency, etc.

We observed no significant differences ($P < 0.05$) between the multivitamin corn-fed group and control groups in terms of absolute organ weights, or organ weights relative to brain or body weights, changes in which often signify hepatocellular, myocardial, adrenal gland and renal tubular hypertrophy, neurotoxicity in the brain and toxicity-related alterations in reproductive or lymphoid organs. For these assays, we measured the weights of the adrenals, brain, epididymis, heart, kidneys, liver, ovaries, spleen, testes thymus and uterus (supporting information Tables S2 and S3).

We identified no histopathological anomalies specific to the multivitamin corn diet (supporting information Table S4). The few anomalies observed were distributed among all three animal groups and are commonly observed in rodents of this age and are considered spontaneous. Some of these histopathological events are shown with normal tissue structures for comparison in Figure 3.

Discussion

We had previously reported the development of a prototype multivitamin corn variety based on the South African elite white corn inbred M37W. This corn is simultaneously engineered to accumulate high levels of β -carotene, ascorbate and folate (Zhu *et al.*, 2008; Naqvi *et al.*, 2009). The best performing line

accumulated 57 $\mu\text{g/g}$ dry weight β -carotene (>169-fold increase), 106.94 $\mu\text{g/g}$ dry weight ascorbate (>sixfold increase) and 200 $\mu\text{g/g}$ dry weight folate (twofold increase) in the kernels, and therefore has the potential to address deficiency diseases caused by the lack of vitamins A, B9 and C. A reasonable portion of multivitamin corn (100–200 g) provides the full reference dietary intake (RDI) of β -carotene (as a sole source of vitamin A), more than the RDI of folate and about 20% of the RDI of ascorbate.

All genetically engineered crops intended for consumption by humans must be rigorously tested to ensure safety, with the benchmark set by comparison with an equivalent unmodified variety which is 'generally regarded as safe'. Genetically engineered crops are evaluated to determine acute and sub-chronic toxicity as well as allergenicity. Similar assessments are not required of nontransgenic varieties even if these are genetically identical to a transgenic crop but are produced by mutation or conventional breeding. They are not required even if the conventional crop is generated by crossing to wild species that are known to contain toxins or allergens. The double standards applied to genetically engineered and conventional crops have the unfortunate consequence that engineered crops such as Golden Rice, which could provide a real and immediate benefit to poor populations in developing countries riven by micronutrient deficiency diseases, are held up in a regulatory quagmire and lives continue to be lost unnecessarily (Potrykus, 2010).

The aim of acute or sub-acute toxicity assessment is to identify immediate or short-term toxic effects, whereas the aim of sub-chronic toxicity evaluation is to determine any adverse effects caused by repeated exposure for a longer period. EFSA guidelines recommend that risk assessments should include at

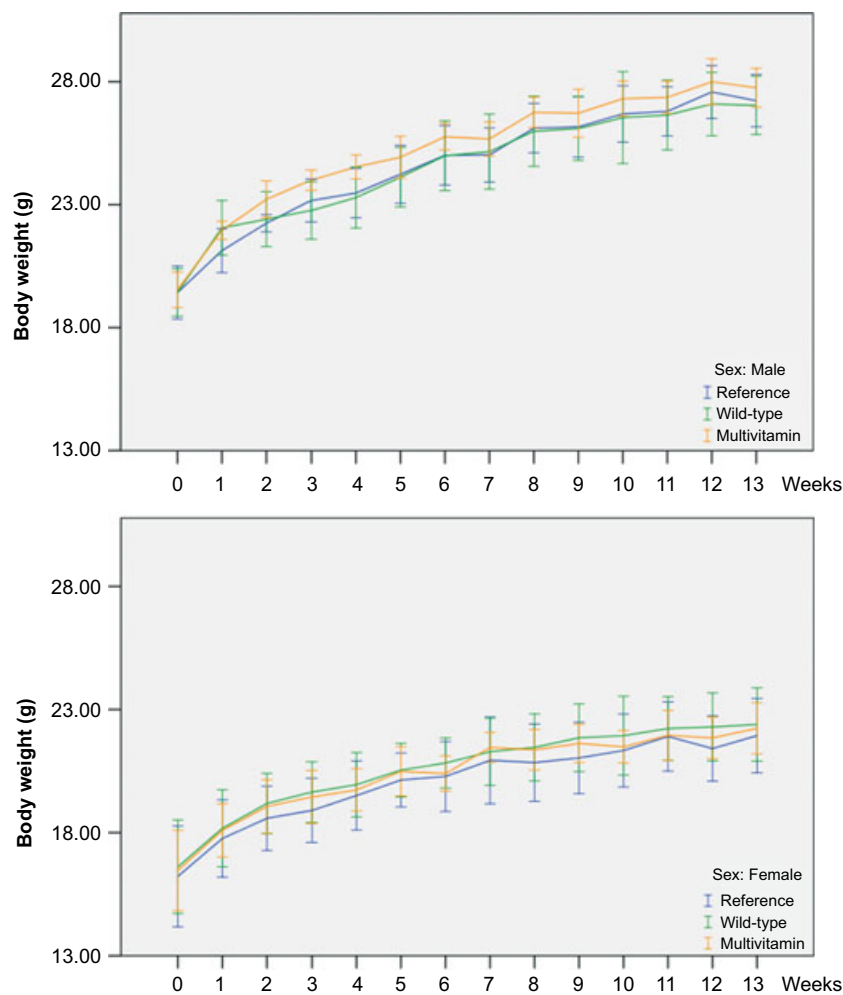


Figure 2 Mean weekly body weights (mean $\pm$ confidence interval 95%) of male and female mice. Animals were fed rodent diet (reference group), and rodent diets containing wild type and multivitamin corn for 13 weeks.

least a 90-day toxicity assessment on whole food/feed in rodents if the composition of the engineered crop is modified substantially or if there are indications for possible unintended effects (European Food Safety Authority, 2011a). However, these requirements have now become de facto standards for engineered crops even if there is no evidence of potential adverse effects and are recommended in Europe to increase consumer confidence that food/feed from engineered crops is as safe as a conventional counterpart (European Food Safety Authority, 2011b).

Our compositional analysis clearly showed no nutritionally relevant differences between the multivitamin corn and the control corn in terms of major constituents, and indeed, the only difference was the expected higher levels of β -carotene, folate and ascorbate in the multivitamin corn. These nutritional differences represent the intended effects of the genetic modification and therefore do not constitute ‘unintended effects’ that the safety tests are ostensibly designed to identify. Having established the substantial equivalence of multivitamin and control corn, we carried out a 28-day assessment to evaluate the

Table 1 Biochemistry mean values $\pm$ SD for males

	Group		
	Reference	Wild type	Multivitamin
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
ALT (U/L)	84 $\pm$ 27	79 $\pm$ 24*	132 $\pm$ 46*
BUN (ng/L)	0.224 $\pm$ 0.012	0.223 $\pm$ 0.035	0.215 $\pm$ 0.017 [†]
Total cholesterol (mg/dL)	152 $\pm$ 24	148 $\pm$ 11	141 $\pm$ 10 [†]
Total protein (g/dL)	4.9 $\pm$ 0.6	4.9 $\pm$ 0.2	4.8 $\pm$ 0.3*
Albumin (g/dL)	2.99 $\pm$ 0.32	3.07 $\pm$ 0.18	3.11 $\pm$ 0.23*

*N = 5; [†]N = 4.

ALT, alanine aminotransferase; BUN, blood urea nitrogen.

Table 2 Biochemistry mean values $\pm$ SD for females

	Group		
	Reference	Wild type	Multivitamin
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
ALT (U/L)	136 $\pm$ 70	108 $\pm$ 40	135 $\pm$ 65
BUN (ng/L)	0.246 $\pm$ 0.053	0.246 $\pm$ 0.038	0.224 $\pm$ 0.045
Total cholesterol (mg/dL)	118 $\pm$ 18	115 $\pm$ 14	128 $\pm$ 19
Total protein (g/dL)	4.5 $\pm$ 0.3	4.4 $\pm$ 0.3	4.6 $\pm$ 0.3
Albumin (g/dL)	3.12 $\pm$ 0.13	2.99 $\pm$ 0.15*	3.11 $\pm$ 0.28

**N* = 5.

ALT, alanine aminotransferase; BUN, blood urea nitrogen.

palatability of the diets and identify any potential for sub-acute toxicity. There were no statistically significant differences in food consumption, body weight, haematological and biochemical values and organ weights between mice reared on the reference diet, wild type corn diet and multivitamin corn diet, and there were no measurable differences in histopathology.

We then initiated a 90-day sub-chronic toxicity evaluation to determine the effects of repeated exposure to the diet over a period long enough for toxicity to become apparent but too short for age-related effects to interfere with the results. As in the 28-day assessment, the three groups were indistinguishable, showing similar food consumption rates, body weights and body weight gains. Food consumption and body weight are usually measured weekly in feeding trials as these parameters are health and well-being indicators, as well as highlighting unintended nutritional effects. The weight gain we observed was within the normal range for animals of this age. The mean consumption of multivitamin corn in this trial was $\sim$ 57 g/kg body weight/day, which is approximately 210-fold more than the safety margin in humans ($\sim$ 0.27 g/kg body weight/day; DEEM™, 2002).

There were no significant differences between the groups in terms of clinical observations, haematological parameters, biochemical markers and absolute or relative organ weights. Histopathology revealed a similar prevalence of anomalies among the three groups which are regarded as spontaneous, in agreement with data from previous feeding trials involving crops engineered for herbicide tolerance and pest resistance (Appenzeller *et al.*, 2009; Hammond *et al.*, 2004, 2006; Healy *et al.*, 2008).

In conclusion, the genetically engineered multivitamin corn did not show any unintended effects in animal feeding trials designed to evaluate sub-acute and sub-chronic toxicity, tests that although are not mandated by law in most jurisdictions will facilitate public acceptance of multivitamin corn for human consumption. Diets prepared with multivitamin corn were palatable, nutritious and safe in animals, which should pave the way for human trials and the eventual deployment of multivitamin corn in developing country settings to help combat simultaneous multiple micronutrient deficiencies among populations that subsist on a predominantly cereal-based diet.

Table 3 Haematology mean values $\pm$ SD for males

	Group		
	Reference	Wild type	Multivitamin
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
WBC ($10^3/\mu\text{L}$)	5.80 $\pm$ 1.07*	5.94 $\pm$ 1.36	4.97 $\pm$ 1.45
Neutrophils ($10^3/\mu\text{L}$)	0.92 $\pm$ 0.22*	0.96 $\pm$ 0.26	0.75 $\pm$ 0.22*
Lymphocytes ($10^3/\mu\text{L}$)	4.69 $\pm$ 0.90*	4.73 $\pm$ 1.11	4.09 $\pm$ 0.60†
Monocytes ($10^3/\mu\text{L}$)	0.13 $\pm$ 0.06	0.21 $\pm$ 0.10	0.13 $\pm$ 0.12
Eosinophils ($10^3/\mu\text{L}$)	0.04 $\pm$ 0.02	0.03 $\pm$ 0.02	0.03 $\pm$ 0.03
Basophils ($10^3/\mu\text{L}$)	0.04 $\pm$ 0.08	0.01 $\pm$ 0.00	0.02 $\pm$ 0.03
RBC ($10^6/\mu\text{L}$)	9.88 $\pm$ 0.52*	10.14 $\pm$ 0.24	9.91 $\pm$ 0.35
HGB (g/dL)	15.12 $\pm$ 0.79*	15.57 $\pm$ 0.24	15.34 $\pm$ 0.56*
HCT %	43.54 $\pm$ 1.79*	44.93 $\pm$ 1.02	44.02 $\pm$ 1.29
MCV (fL)	44.45 $\pm$ 0.99	44.32 $\pm$ 0.22	44.42 $\pm$ 0.44
MCH (pg)	15.33 $\pm$ 0.28	15.35 $\pm$ 0.22	15.38 $\pm$ 0.19*
MCHC (g/dL)	34.48 $\pm$ 0.90	34.65 $\pm$ 0.50	34.70 $\pm$ 0.42*

**N* = 5; †*N* = 4.

WBC, white blood cell count; RCB, red blood cell count; HGB, haemoglobin; HCT, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration.

Table 4 Haematology mean values $\pm$ SD for females

	Group		
	Reference	Wild type	Multivitamin
	Media $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
WBC ($10^3/\mu\text{L}$)	5.34 $\pm$ 1.21	6.32 $\pm$ 2.65	5.52 $\pm$ 1.45
Neutrophils ($10^3/\mu\text{L}$)	0.67 $\pm$ 0.21	0.83 $\pm$ 0.20	0.85 $\pm$ 0.24
Lymphocytes ($10^3/\mu\text{L}$)	4.09 $\pm$ 0.99	3.81 $\pm$ 1.00*	4.15 $\pm$ 1.19
Monocytes ($10^3/\mu\text{L}$)	0.53 $\pm$ 0.14	0.62 $\pm$ 0.28	0.48 $\pm$ 0.14
Eosinophils ($10^3/\mu\text{L}$)	0.02 $\pm$ 0.02	0.04 $\pm$ 0.03	0.03 $\pm$ 0.03
Basophils ($10^3/\mu\text{L}$)	0.03 $\pm$ 0.03	0.07 $\pm$ 0.14	0.01 $\pm$ 0.00
RBC ($10^6/\mu\text{L}$)	9.48 $\pm$ 0.26	9.41 $\pm$ 0.31	9.56 $\pm$ 0.32
HGB (g/dL)	15.07 $\pm$ 0.40	15.08 $\pm$ 0.42	15.33 $\pm$ 0.55
HCT %	42.25 $\pm$ 1.16	42.00 $\pm$ 1.24	42.50 $\pm$ 1.52
MCV (fL)	44.58 $\pm$ 0.17	44.65 $\pm$ 0.23	44.45 $\pm$ 0.40
MCH (pg)	15.90 $\pm$ 0.37	16.03 $\pm$ 0.27	16.03 $\pm$ 0.18
MCHC (g/dL)	35.67 $\pm$ 0.70	35.93 $\pm$ 0.60	36.07 $\pm$ 0.42

*N = 5.

WBC, white blood cell count; RCB, red blood cell count; HGB, haemoglobin; HCT, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration.

Experimental procedures

Plant material

Multivitamin corn was created by the stable transformation of the South African inbred M37W with corn *psy1* (phytoene synthase), *Pantoea ananatis crtI* (carotene desaturase), rice *dhar* (dehydroascorbate reductase) and *Escherichia coli folE* (GTP cyclohydrolase) under the control of various endosperm-specific promoters, together with the selectable marker gene *bar* (Naqvi *et al.*, 2009). The corn used for the feeding experiments was from the T7 homozygous generation.

Compositional analysis of experimental and control diets

Multivitamin and control corn plants were grown in the same greenhouse under exactly the same conditions over the same growing period. Vitamin content was measured in a minimum of 10 individual plants by HPLC analysis, and data were analysed by ANOVA.

Experimental diets containing multivitamin corn and control diets containing wild type M37W corn were prepared from freeze-dried powdered kernels under hygienic conditions. Meals were prepared by mixing 2014 Teklad Global 14% Protein Rodent Maintenance Diet (Harlan Laboratories, Madison, WI) with the appropriate freeze-dried corn powder as a 60 : 40 ratio (w/w).

The composition of the corn was compared by quantifying basic chemical parameters such as moisture, fat, ash, crude fibre and protein, as well as the target micronutrient levels (β -carotene, ascorbate and folate levels) (AOAC International, 2000). Moisture was obtained as the loss of weight after drying in an oven at 100 °C to a constant weight. Fat content was estimated using Soxhlet extraction. Ash levels were estimated by gravimetric analysis after ignition in an electric furnace. Crude fibre was determined from the difference between the weight of the residue remaining after the sample was digested under specific conditions and the weight of the ash. Protein was estimated through total nitrogen content by the Dumas

combustion method. Protein content was calculated by applying a nitrogen-to-protein conversion factor of 6.25.

The β -carotene, ascorbate and folate levels were determined as described in (Naqvi *et al.*, 2009).

Animal feeding studies

The experimental design was adapted from OECD guidelines 408 and 409 for the 28- and 90-day studies respectively, following EFSA recommendations (European Food Safety Authority, 2006). The study complied with Law 5/1995 and Act 214/1997 of the Autonomous Community (Generalitat) of Catalonia and EU Directives (EEC 63/2010) and was approved by the Ethics Committee on Animal Experiments of the University of Lleida.

Male and female albino BALB/c mice obtained from Harlan Laboratories Models S.L. (Sant Feliu de Codines, Spain) were acclimated in individual cages 1 week before the experiment. The mice had *ad libitum* access to a standard 2014 Teklad Global 14% Protein Rodent Maintenance Diet and water. The animal rooms were environmentally controlled and maintained at 20 $\pm$ 2 °C, relative humidity 50 $\pm$ 5%, with a 12-h photoperiod.

Following acclimation, 6-week-old animals were randomly assigned to three groups of 12 (six male and six female) excluding animals deviating significantly from the group mean body weight ($P < 0.05$). Fresh food was supplied 2–3 times per week as required, for 90 days. The reference group continued to be fed with the standard 2014 Teklad Global 14% Protein Rodent Maintenance Diet after acclimation. The control group was fed with a 40 : 60 mixture of powdered wild type corn kernels and the reference diet. The experimental group was fed with a 40 : 60 mixture of powdered multivitamin corn kernels and the reference diet.

Clinical observations

Clinical observations were recorded before the experiment and daily while the experiment was in progress. No moribund or

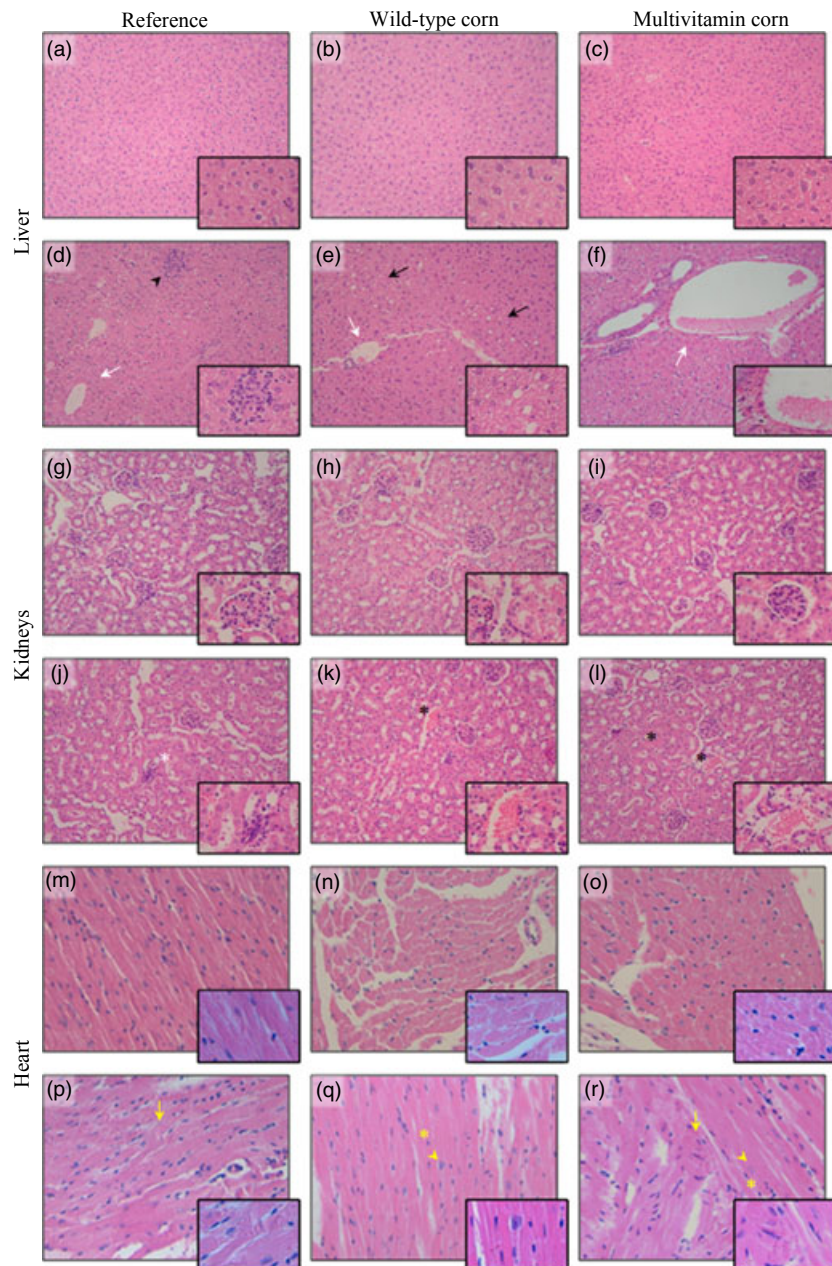


Figure 3 Histology (haematoxylin and eosin staining) from liver (a–f) and kidneys (g–l) $\times 20$ ($\times 40$ in the box) and heart (m–r) $\times 40$ ($\times 63$ in the box); (a) (b) and (c) showing normal liver structure from reference, wild type and multivitamin groups, respectively; (d–f) showing lymphoid aggregates (black arrowhead), sinusoid congestion and/or central vein dilatation (white arrow) and steatosis (black arrow); (g) (h) and (i) showing normal kidney structure from reference, wild type and multivitamin groups, respectively; (j–l) showing lymphoid aggregates (white *) and red blood cells in the interstitium (black *); (m), (n) and (o) showing normal heart structure from reference, wild type and multivitamin groups, respectively; (p–r) showing contraction bands (yellow arrow) and hypertrophic cells (yellow arrowhead) with increased nuclear size in comparison with normal cell (yellow *).

dead animals, abnormal behaviour, abnormal appearance or abnormal droppings were observed. We also measured the feed intake and body weight of all mice on a weekly basis.

We carried out biochemical and haematological tests on blood samples taken from the submandibular vein while the animals were under isoflurane anaesthesia preceding the terminal sacrifice. Whole blood was collected in a tube containing EDTA as an anticoagulant, and plasma was prepared by centrifugation at 1500 g for 10 min prior to the analysis of alanine aminotransferase (ALT), total cholesterol, total protein, albumin and blood urea nitrogen (BUN) using a Hitachi Modular analyzer (Roche, Badalona, Spain). We also measured the white blood cell count (WBC), differential white blood cell count of neutrophils, lymphocytes, monocytes, eosinophils, basophiles, red blood cell count (RCB), haemoglobin (HGB), haematocrit (HTC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH) and mean

corpuscular haemoglobin concentration (MCHC) using a Sysmex XE-5000 analyzer (Roche).

We also carried out autopsies on all animals and determined the weight of adrenals, brain, epididymides, heart, kidneys, liver, ovaries, spleen, testes, thymus and uterus (paired organs were weighed together). The adrenals, aorta, caecum, cervix uteri, colon, duodenum, epididymides, eyes, heart, ileum, jejunum, kidneys, liver, lungs, oesophagus, ovaries, pancreas, Peyer's patches, prostate, rectum, sciatic nerve, seminal vesicle, spinal cord, spleen, sternum (bone marrow), stomach, bone and muscle (from femur and quadriceps), testes, thymus, thyroid/parathyroid, trachea, urinary bladder, uterus, vagina, biliary vesicle and skin were then fixed, dehydrated, embedded in paraffin, sectioned (4–5 μ m) and stained with haematoxylin–eosin. The adrenals, brain, epididymides, heart, kidneys, liver, lungs, ovaries, pancreas, spleen, testes, thymus, thyroid/parathyroid

and uterus of all animals were subject to histopathological analysis as these represent the major target organs for toxicity effects, and the remaining samples were reserved for analysis in the event of abnormal histopathology of these initial specimens.

Statistical analysis

We compared animal weights, feed consumption, biochemical data, haematological data and organ weights between the multivitamin corn diet group and the reference diet group, and (separately) between the multivitamin corn diet group and the control corn diet group using PASW Statistics 18 (Version 18.0.0, 2009). The data were also analysed separately in male and female groups. Differences were considered statistically significant at $P < 0.05$.

We carried out preliminary homogeneity of variances (Levene's Test) and normality of data distributions (Q-Q plots, histograms and Shapiro–Wilk tests) on all data (continuous values). If these tests were not statistically significant, we assumed normal distribution, and we carried out analysis of variance (ANOVA) to compare the multivitamin corn diet group with both controls (Student's t -test applying the Bonferroni method of correction for multiple comparisons). If one of the preliminary tests was significant, we carried out nonparametric tests using Kruskal–Wallis for k independent samples, and if this yielded a significant result, we evaluated the contrasting data case-by-case using the Mann–Whitney test. For multiple comparisons, we applied the Bonferroni method of adjustment to maintain a 5% type error rate per response variable in both bivariate tests.

Acknowledgements

This work was supported by the Ministry of Science and Innovation, Spain (BFU2007-61413 and BIO2007-30738-E) and European Research Council Advanced Grant (BIOFORCE) to PC. We thank Ana Martínez and Laura Arcal for excellent technical assistance and the entire team in the laboratory of Applied Plant Biotechnology at the Universitat de Lleida for stimulating discussions.

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Supporting information

Additional supporting information may be found in the online version of this article:

Table S1 Proximates of corn and experimental diets (^amicrograms per gram DW).

Table S2 Final body weight and absolute organ mean values $\pm$ SD for males.

Table S3 Final body weight and absolute organ mean values $\pm$ SD for females.

Table S4 Histopathological analyses for male and female mice.

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Supporting information tables

Table S1

Diet composition (%)	Wild-type corn grain	Multivitamin corn grain
Moisture	5,21	5,89
Fat	3,75	4,36
Ash (% DW)	2,09	2,07
Crude fiber	4,05	4,58
Protein	14,62	16,75
β -carotene ^a	0,35	59,32
Ascorbate ^a	17,53	106,94
Folate ^a	0,93	1,94

Table S2

	Group		
	reference	wild-type	multivitamin
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
Final body weight	27,23 $\pm$ 1,01	27,04 $\pm$ 1,13	27,76 $\pm$ 0,75
Adrenals (g)	0,0033 $\pm$ 0,0013	0,0033 $\pm$ 0,0016	0,0027 $\pm$ 0,0006
Brain (g)	0,3110 $\pm$ 0,0341	0,3352 $\pm$ 0,0476	0,3404 $\pm$ 0,0480
Epididymis (g)	0,1055 $\pm$ 0,0076	0,1093 $\pm$ 0,0122	0,1112 $\pm$ 0,0087
Heart (g)	0,1502 $\pm$ 0,0082	0,1479 $\pm$ 0,0077	0,1493 $\pm$ 0,0062
Kidneys (g)	0,4070 $\pm$ 0,0431	0,3975 $\pm$ 0,0322	0,3907 $\pm$ 0,0308
Liver (g)	1,3006 $\pm$ 0,1120	1,2889 $\pm$ 0,0514	1,2516 $\pm$ 0,1447
Spleen (g)	0,0707 $\pm$ 0,0132	0,0876 $\pm$ 0,0119	0,0822 $\pm$ 0,0039
Testes (g)	0,2169 $\pm$ 0,0225	0,2223 $\pm$ 0,0054	0,2153 $\pm$ 0,0123
Thymus (g)	0,0303 $\pm$ 0,0021	0,0299 $\pm$ 0,0050	0,0318 $\pm$ 0,0092

Table S3

	Group		
	reference	wild-type	multivitamin
	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
Final body weight	21,94 $\pm$ 1,44	22,39 $\pm$ 1,42	22,24 $\pm$ 0,99
Adrenals (g)	0,0054 $\pm$ 0,0015	0,0061 $\pm$ 0,0014	0,0062 $\pm$ 0,0005
Brain (g)	0,3313 $\pm$ 0,0315	0,3299 $\pm$ 0,0137	0,3305 $\pm$ 0,0302
Heart (g)	0,1135 $\pm$ 0,0093	0,1167 $\pm$ 0,0096	0,1148 $\pm$ 0,0093
Kidneys (g)	0,2654 $\pm$ 0,0325	0,2629 $\pm$ 0,0296	0,2557 $\pm$ 0,0024
Liver (g)	1,0116 $\pm$ 0,0914	1,0126 $\pm$ 1,0639	1,0203 $\pm$ 0,0901
Ovaries (g)	0,0130 $\pm$ 0,0027	0,0106 $\pm$ 0,0027	0,0111 $\pm$ 0,0022
Spleen (g)	0,0833 $\pm$ 0,0064	0,0947 $\pm$ 0,0084	0,0832 $\pm$ 0,0110
Thymus (g)	0,0274 $\pm$ 0,0036	0,0272 $\pm$ 0,0041	0,0293 $\pm$ 0,0058
Uterus(g)	0,0708 $\pm$ 0,0384	0,0609 $\pm$ 0,0130	0,0778 $\pm$ 0,0054

Table S4

		Males			Females		
		Refer ence	Wild- type	Multivit amin	Refer ence	Wild- type	Multivit amin
epididymides	oligospermia	1	0	0	-	-	-
heart	hypertrophy	4	4	3	1	3	3
	contraction band	1	3	1	2	0	0
kidneys	lymphoid aggregates	1	1	3	1	0	1
	extravasated red blood cells in the interstitium	6	6	6	5	6	6
liver	sinusoid congestion and central vein dilatation	6	6	6	6	6	6
	steatosis	5	4	4	0	0	1
	inflammatory cells and necrotic debris	1	3	4	6	5	6
	iron deposits	1	1	0	3	2	2
	apoptotic cells	1	0	0	0	0	0
spleen	pigment, increased	5	6	6	6	6	6
thyroid/parathyroid	cyst change	0	0	0	1	0	0