

Bt brinjal Event EE-1
The Adequacy of the GEAC Risk Assessment: Its impacts on Human and Animal
Health and India's Food Security

Bt brinjal Toxicology Assessment
Review of 90-day Subchronic Oral Toxicity in Rats

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SUMMARY

This evaluation of Bt brinjal studies is based on requirements for a rigorous evaluation of food safety for the people of India and their health. Departures from Indian and international published standards for the 14-day and 90-day studies are sufficient to cause alarm¹.

The current food safety studies for Bt brinjal were not conducted in accordance with published standards, did not accurately summarize results, and ignored toxic endpoints for rats fed Bt brinjal: in particular, rats fed a Bt brinjal for 78 out of 90 days (only one dose level) experienced:

- organ and system damage: ovaries at half their normal weight, enlarged spleens with white blood cell counts at 35 to 40 percent higher than normal with elevated eosinophils, indicating immune function changes.
- toxic effects to the liver as demonstrated by elevated bilirubin and elevated plasma acetylcholinesterase

Major health problems among test animals were ignored in these reports. The single test dose used was lower than recommended by the Indian protocols. Release of Bt brinjal for human consumption cannot be recommended given the current evidence of toxicity to rats in just 90 days and the serious lack of scientific integrity surrounding the reports.

Unanswered Concerns regarding the safety assessment of Bt Brinjal:

Neurological function, behavioral effects, reproductive performance and biological resilience of test animals were not evaluated in these studies. Further research is needed to address these important endpoints that showed signs of weakness in the current studies.

Dietary equivalence of dried brinjal, dried Bt-brinjal and control diets was not addressed. Concentrations of the new insecticide protein Cry1A(c) were not measured in dried brinjal powder. It is important to know how much of this new protein was actually in the dried samples fed to the rats, especially since there is data to suggest that Cry1A(c) is at least partially destroyed in laboratory heating conditions. However, this was not done. That omission makes it impossible to compare the test diet with insecticide concentrations expected in cooked human food.

¹ The Indian toxicology standards for 14-day and 90-day feeding trials published by the Department of Biotechnology (DBT) in 1998 and in 2008 fall short of the international standards (OECD 1998 and Codex Alimentarius 2003a-c), allowing a significant loss of scientific rigour. Therefore, although this critique is based on the Indian DBT protocol, meaningful departures and omissions from international standards are noted. It is important to clarify that 14 and 90 day exposures to rodents are insufficient periods of time on which to base food safety decisions for humans.

The use of laboratory animals to test food safety, although widely accepted as a toxicological tool, is only an indication of effects that might be expected from human exposure²; deviations from standard protocols need to be evaluated carefully. Yet every departure made by INTOX (the laboratory contracted to do the research) from the Indian Department of Biotechnology protocol (1998) has resulted in lower standards being used, with less power to detect changes experienced by rats eating Bt brinjal. These include: skipping important endpoints such as IgE measurement to test for allergenicity, testing only one dose that was lower than human consumption is likely to be, ignorance of toxicological equivalence, lost data, lack of Good Laboratory Practice standards, inadequate observation of animals, a 29% decrease in exposure days in one study (doses were administered 5 days per week instead of 7), etc.

Summary of statistically significant findings in rats fed Bt brinjal over 90 days with implications for human health		
Indicator	What it might indicate	Significant potential adverse effect
Elevated white blood counts from chronic exposure	Inflammation, allergy, tissue injury	√
Higher aspartate aminotransferase in blood from acute exposure	Liver damage	√
Elevated bilirubin in blood		
Altered plasma acetylcholinesterase		√
Smaller ovaries	Reproductive toxicity	√
Enlarged spleens	Chronic infections or blood cancer	√

Consequently, the studies submitted by the applicant company are woefully inadequate to determine the safety of Bt brinjal for long-term human consumption.

² The use of animals to test food safety introduces uncertainty in risk assessment that cannot be overcome without imposing unknown risks on human individuals instead. Significant genetic and phenotypic variation between humans makes it statistically impractical to conduct food safety trials on humans.

METHODS

The current assessment is of three toxicology studies conducted by commercial toxicology laboratory INTOX PVT LTD on behalf of Maharashtra Seed Company, also known as Mahyco. Study details and raw data have recently been made available to the public through the internet link http://www.envfor.nic.in/divisions/csurv/geac/bt_brinjal.html.

All three studies tested the genetically modified food brinjal (herein referred to as Bt brinjal)³ containing the insecticide protein Cry1A(c) and other novel genetic components, as it is proposed for sale of seed in India;

- a 14-Day Dose Range Finding Study
- an Acute Oral Toxicity study of Transgenic Bt brinjal containing Cry1A(c) gene in rat (Study No. 218301)
- a 90-day Subchronic Oral Toxicity Study of Transgenic Bt brinjal (Study No. 218304)

These studies are herein referred to as the 'dose-range finding', the '14-day' and the '90-day' studies, respectively.

The dose-range finding study

The first of these published studies is a 14 day dose-range finding study of Bt brinjal in rats. Although this study was submitted by the GM company, it is misleading to do so as it occurs within a report for another study. Only limited information about the study is provided, and then only in summary form on pages 12 and 13 of the 90-day study report, as well as some raw data in Appendix D. This study is disregarded from further consideration for the following reasons:

- Only three animals were tested per dose group, which is insufficient to make any valid conclusions. According to the Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds and Plant Parts (DBT 1998), a minimum of ten animals per dose group is necessary. This is considerably less than the OECD standard of ten animals per sex and dose group.
- The problem with using fewer than the recommended number of animals is an increased chance of Type II errors – that is, failing to observe a treatment-related difference when in truth there is one.

In addition to using too few animals to provide confidence in the findings, there were other arbitrary and unjustified methodological practices:

- The rationale for using doses of dried brinjal powder at 500 and 1000 mg/kg was not provided.

³ Brinjal is also known as eggplant or aubergine

- The study guidelines and laboratory standards for this study were not provided. Statements about following good laboratory practice (GLP) or having GLP certification are also absent. Lack of stated adherence to laboratory standards puts the quality of the research conducted into question.
- The dates of the study and the names, titles and signatures of the people conducting the study were not provided.

The 14-day and 90-day studies

The 14-day and the 90-day studies are stated to have been conducted according to “Revised Guidelines for Research in Transgenic Plants and Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds and Plant Parts”⁴ as outlined by the Department of Biotechnology (DBT) in India in 1998, and in compliance with the principles of Good Laboratory Practice as established by the OECD in 1998.

Statements of compliance with Good Laboratory Practices and Quality Assurance (pages 3 and 4 of both study reports) are not signed. This omission does not inspire confidence in the published results.

Inclusion of extra control groups

Only one control group was required according to the Indian protocol (DBT 1998, page 61) and international protocols (OECD 1998, Item 14 page3)(Codex Alimentarius 2003b). The applicant company used three control groups for each single dose test group:

- G I (14 day test) and G1 (90 day test): Controls receiving vegetable oil only (vehicle control)
- G II (14 day test) and G2 (90 day test): Vegetable controls receiving non-transgenic brinjal powder in oil
- G III (14 day test) and G3 (90 day test): Vegetable controls receiving commercially available non-transgenic brinjal powder in oil

Was the non-transgenic brinjal group included under the assumption that the studies would find no toxicity at the doses used [5000 mg/kg-day in the 14-day study and 1000 mg/kg-day in the 90-day study], and therefore suffice as the ‘limit tests’ described on pages 54/55 and 62 of the protocol (DBT 1998)? The second and third control groups listed above were not required for the 14-day or the 90-day studies.

⁴ Herein referred to as ‘the protocol’

Such an inclusion of extraneous control groups is not scientifically or methodologically valid. Increasing the number of control groups in this manner decreases the chances that differences will be consistently observed between the Bt brinjal group and others.

The salient analysis of toxicology results is between equal numbers of individuals from the Bt brinjal group and a single control group. Since it is unnecessary to produce more than one control group, commercially available brinjal dose groups (G III and G3) are not considered further in this analysis.

Presence or absence of Cry1A(c) protein in brinjal powder

No effort was made to confirm that the commercially-available brinjal did not contain the Cry1A(c) protein, or other agricultural chemicals that may adversely affect the health of animals eating it.

Were fruit powders received from Mahyco verified for the presence or absence of transgenic material just prior to conducting toxicity tests? The only evidence we have that testing was conducted to confirm the presence of Cry1A(c) protein in Bt brinjal and non-Bt brinjal in these studies is a single page that was produced twice: at the end of the 14-day study report (no page number) and also as Appendix E on page 106 of the 90-day study report. Since there is no date on this page and these two studies were conducted more than one year apart, it is impossible to know which study it was produced for.

Evidence of testing for the Cry1A(c) protein in animal feed is *either* misrepresented in one or both of these reports *or* both studies used the same stored batches of dried brinjal powder. The possibility that transgenic proteins degraded during drying or after storage cannot be ruled out, representing a significant potential loss of potency of the test article. Furthermore, there is no indication of the concentrations of Cry1A(c) protein in dried brinjal powder either before or after several months of storage. In turn, this would be a further loss in representation of laboratory tests at a dose that consumers are likely to be exposed to.

New Statistical Analyses

Raw data from the published reports were used to calculate statistically significant differences between test groups using a student's t-test for two independent samples with unequal variance using Microsoft Office Excel 2007. The raw data selected were variables noted from visual inspection of the summary tables for each report. This included concentrations of acetylcholinesterase (a neurotransmitter enzyme) from plasma and red blood cells, bilirubin (increases indicate liver complications from infection or chemical exposure), total white blood cells (increased in response to infection) and aspartate aminotransferase (increases are used to diagnose liver or heart damage) in blood. In the 90-day study, organ weights for ovaries (which give an indication of reproductive health), spleen (this organ purifies the blood) and kidneys (which excrete waste products from the body) were also analysed.

Direct statistical comparisons in both studies are made between the main test group (G IV and G4, receiving Bt brinjal powder in peanut oil) and the group receiving peanut oil only (G I and G1).

Comparisons between the Bt-brinjal test groups and the control group receiving non-transgenic brinjal in peanut oil (G II and G2) are described in the text and noted in Appendix B of this report.

This report addresses the following questions:

1. Do the two studies meet the stated 1998 protocol standards⁵ for India and the OECD standard?
2. Have the studies been accurately summarized to be consistent with the raw data results? Were statistical assumptions valid and adequately described?
3. Would an impartial technical reviewer derive the same conclusion as the laboratory contracted by the seed company (and accepted by the second Expert Committee or EC II)?

The larger question of whether or not these results are sufficient to draw conclusions of food safety is addressed in the Discussion section of this report.

⁵ The DBT protocol was updated in 2008. Since this research was conducted prior to 2008, the 1998 protocol was relevant at the time. Neither of these protocols adhere to international standards.

RESULTS

Table 1 below summarizes the compliance of the 14 day acute toxicity test and the 90 day feeding study with the stated guidelines (DBT 1998).

Table 1. Summary of Study Characteristics in Compliance with Protocol Guidelines (1998)⁶

Protocol Requirement, Department of Biotechnology 1998	14 day study	90 day study
Sufficient number of animals tested per dose group; 10 for 14 day study and 20 for 90-day study	Yes	Yes
Animals housed singly or in pairs (not a protocol requirement for 90-day study)	Yes	No
Test doses selected according to protocol	Yes	No
Daily (twice daily required for 14-day study) observations of animals to look for signs of toxicity including tremors, convulsions, salivation, diarrhea, lethargy and sleep, dyspnea, coma, nasal bleeding, etc ⁷ .	Undetermined	No
Daily observations of behavioral abnormalities	No	No
Statistical methods described	No	Yes
Statistical methods used	No	No
Statistical results reported	No	No
Significant differences discussed in terms of biological significance and impact on food safety	No	No
Study summary reflects results	No	No

⁶ Revised Guidelines for Research in Transgenic Plants and Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds and Plant Parts. Department of Biotechnology, Ministry of Science and Technology, Government of India, August 1998. Public Printing Service (Delhi) 96 pp.

⁷ Updated protocol for 2008 emphasizes the importance of behavioural signs of toxicity not limited to hunched posture, lethargy or persistent recumbancy, labored breathing, any condition interfering with eating or drinking (e.g., difficulty moving), or excessive or prolonged hyperthermia or hypothermia.

The 14 Day Acute Oral Toxicity of Transgenic Bt brinjal containing Cry1A(c) gene in rat

An acute oral toxicity study (a limit test) was performed on rats fed 5 grams of dried brinjal powder per kg of body weight in peanut oil. Doses were administered over 24 hours and rats were observed for 14 days following dosing.

As shown in Table 1, the 14 day study was conducted with several deviations from the 1998 DBT protocol: the report lacks a description of statistical methods used, study results were not compared using a statistical analysis, and important variations in health endpoint outcomes were not discussed in terms of biological significance. These are critical and unjustifiable omissions by the researchers. Consequently, while the study has been used by the GM crop company to provide evidence that Bt brinjal is safe to eat, this conclusion cannot be substantiated.

New Statistical Comparisons

For the purposes of verifying the conclusions reported in the 14 day study, the following statistical comparisons have been made on endpoints of interest⁸ from the following sources in the INTOX report:

- Appendix B1 of INTOX report: Individual animal hematology data
- Appendix B2 of INTOX report: individual animal clinical chemistry data

⁸ Endpoints of interest were selected from quick visual inspection of data summary tables. This is not an exhaustive analysis of all raw data from the 14-day report.

Table 2. Results of statistical analysis of raw data from the 14 day study

Toxicological endpoint	Arithmetic mean values for females/males/total	
	Vehicle control group (G I)	Bt brinjal group (G IV)
Total white blood cells (x10 ³ /cmm) females/males/total	8.6/9.0/8.8	7.7/8.2/8.0
Aspartate aminotransferase (IU/L) females/males/total	164.2/154.0/159.1	251.8**/244.8*/248.3**
Plasma acetylcholinesterase (IU/L) females/males/total	641.8/656.2/649.0	534.0/529.3/531.7**
Red blood cell acetylcholinesterase (IU/L) females/males/total	407.6/398.8/403.2	351.9/324.9/338.4
Bilirubin (mg/dl) females/males/total	1.1/0.9/1.0	1.1/1.2*/1.2*

*Statistically significant difference from G IV at $p \leq 0.05$

**Statistically significant difference from G IV at $p \leq 0.01$

Toxicological implications of the lab results in Table 2 from 14 day study

Total white blood cell counts were found to be 9 to 12% lower among the rats fed Bt brinjal compared to controls. The toxicological implications of *decreased* white blood cell count following an acute exposure include a possible recent infection or impaired immunological function.

Increases in aspartate aminotransferase (AST) among Bt brinjal-fed rats were 54 to 60% higher than controls. Increased AST indicates damage to the liver or heart. In this case, coupled with elevated bilirubin (another measure of liver dysfunction also noted in this table), damage to the liver from short-term exposure at the dose of 5000 mg/kg-day is indicated.

Plasma acetylcholinesterase was 22% lower among rats fed Bt brinjal than that observed for controls. Significant changes in plasma acetylcholinesterase (a neurotransmitter enzyme) concentrations could be further evidence of liver damage in rats fed Bt brinjal.

Inconsistencies in the 14-day study report

Page 6 of the report is a summary of the 14-day study. In this summary there are only three study groups mentioned as follows: "...the test article was administered orally to a group of 5 male and 5 female rats as an acute dose at the limit dose of 5000 mg/kg body weight, suspended in peanut oil, as a vehicle. One concurrent control group of 5 male and 5 female rats was similarly gavaged with nontransgenic brinjal powder in peanut oil, while a third group of 5 male and 5 female rats was gavaged with normal powdered rodent diet in peanut oil only, and served as an untreated control." The study summary appears to be at odds with the data reporting results for four study groups, not three.

Page 9 of the report states: The total number of animals tested per sex is 20 (five per dose group using four dose groups) but the total row table lists that there are 15 animals per sex. These may only be typographical errors. However, they may also indicate that the third test group (G III, not mentioned in the study summary) results was added at a later date.

The results given in Table 3.3 (individual animal fate and pathology findings) of Appendix B (of the INTOX report) for group G III are identical to the results in the next table for Bt brinjal-fed rats among both males and females. This is another suspected formatting/typographical error but with larger implications: where are the missing data for these animals and what information is contained in those missing data?

The protocol requires twice daily observation of animals for signs of toxicity since the test article is given in a single acute dose. Tables A1.1 through A1.4 report no clinical abnormalities over 14 days of observation. It is unlikely that clinical observations would not pick up a single abnormality among 40 rats over 14 days, if observations had been conducted by trained researchers.

The 90-day Subchronic Oral Toxicity Study of Transgenic Bt brinjal

Animal Husbandry

The outcome from caging groups of 5 animals together instead of housing them singly or in pairs is extreme crowding, unless very large cages were used. Group caging also has the effect of "washing out" individual differences in amounts of food and water consumed over the course of the study. If one animal in five has an abnormal eating or drinking pattern (as was the case among goats fed Bt brinjal) this is unlikely to be observed in a group measurement, even though a health outcome for 20 percent of the population (one in five animals) is of interest. It is noted that this is not strictly a deviation from either the 1998 protocol or the OECD 408 protocol which states that animals may be housed in small groups of the same sex in the 90-day study (OECD 1998).

Were all test group animals placed in the same room to minimize differences in temperature, humidity and air changes that will impact on the overall health of the animals? This needs to be specified but was not.

Were all test group animals obtained from the same source, at the same age, previously unexposed to Bt brinjal and nulliparous at the start of the study? Were animals randomly assigned to dose groups at the start of the study? All of these variables need to be reported as they have the potential to affect health outcomes measured in this study, but none of them were.

Dose groups

The 90-day toxicity study is meant to include 3 doses of Bt brinjal. According to the Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds and Plant Parts;

“The selection of the dose is made on the basis of acute toxicity studies of the test chemical. At least 3 dose levels, one maximum, one minimum and one intermediate are used. Consideration is given that the highest dose may result in toxic effects without causing excessive lethality and the lowest dose may not produce any toxic effects. A group of vehicle controls is also used.” –(DBT 1998).

The 90-day study was conducted using a single dose level for which there is no demonstrable toxicology information prior to conducting the study. Without evidence to support the assumption that 1000 mg/kg-day will result in toxic responses in a 90-day study, this particular dose does not make sense scientifically.

One possible outcome of using a dose for which there is no evidence of toxicity would be a false finding of safety because the dose was too small to observe toxic effects in rodents over 90 days. This increases the chance of failing to observe a treatment-related toxic endpoint when in truth there may be one.

Reasons for using the dose of 1000 mg/kg were tacitly given by stating that a dose range finding study had been conducted. Since this test used a total of three animals per test group, and for other reasons listed above, this cannot be considered justification for selecting the 1000 mg/kg-day dose over 90 days.

Moreover, there are justifications for believing that one gram of brinjal per kg body weight is inadequate to determine the health effects of this crop on Indian people. Brinjal is a crop that is widely consumed in significant amounts in India. The dose used in this study is equivalent to only 40g (about 2 tablespoons) of Bt brinjal/day for a slightly-built woman and 70g/day (about 4 tablespoons) for a reasonably-sized man. Notably, Codex (Codex Alimentarius 2003) recommends:

“Information about the known patterns of use and consumption of a food, and its derivatives should be used to estimate the likely intake of the food derived from the recombinant-DNA plant. The expected intake of the food should be used to assess the nutritional implications of the altered nutrient profile both at customary and maximal levels of consumption. Basing the estimate on the highest likely consumption provides assurance that the potential for any undesirable nutritional effects will be detected. Attention should be paid to the particular physiological characteristics and metabolic requirements of specific population groups such as infants, children, pregnant and lactating women, the elderly and those with chronic diseases or compromised immune systems. Based on the analysis of nutritional impacts and the dietary needs of specific population subgroups, additional nutritional assessments may be necessary. It is also important to ascertain to what extent the modified nutrient is bioavailable and remains stable with time, processing and storage.” paragraph 49, Section 1.

This recommendation was clearly not followed for this feeding study.

Other omissions in the 90 day study

IgE was not measured in this study, even though the report states on page 16 that IgE was analysed. Clinical chemistry data in Appendix B2 report IgE results as <1.00 IU/ml for every observation. Since IgE concentrations vary widely between individual rats (Abadie and Prouvost-Danon 1980) and expected values in rats are greater than 200 IU/ml⁹, it is likely that:

- The IgE measurement method used by the researchers using the “Erba Smartlab Random Access Batch Analyser” (page 16 of the report) was not sensitive enough to accurately measure IgE in rats.
- Blood samples were incorrectly stored prior to chemical analysis leading to serious errors in the results.

The lack of IgE data is unfortunate as IgE is especially important in this study as a measure of allergic reactivity. Quantitative evaluation of IgE is required in the protocol on page 62 of DBT 1998. This is a serious omission and protocol deviation that has not been addressed. Without IgE data, there is a serious lack of important information about the possible effects of the Cry1A(c) protein on the mammalian gut resulting in possible hypersensitivity/allergic reactions, observed as increased concentrations of IgE compared to controls. On the other hand, decreased concentrations of IgE in Bt brinjal rats would be consistent with diseases such as hypogammaglobulinemia, autoimmune diseases, ulcerative colitis, and hepatitis. It is important to know whether the new brinjal may simply be an irritant that produces allergic responses in the gut, or an endotoxin that confers damage to the liver with ingestion.

Raw data in Appendix B1 for differential white blood cells are reported in whole numbers without decimal places, preventing analysis of eosinophil concentrations. The summary table for these results (Table 8 on pages 38-39), however, reports concentrations at two decimal places (two more significant digits than the raw data support). Is this a lack of precision in reporting individual raw data, or is it over-precision in aggregate data? It is impossible to tell if the aggregate data actually reflect the raw data in this case. The overall effect of leaving out these important raw data is to prevent independent analysis of differentiated white blood cell counts in rats fed Bt brinjal.

New Statistical Comparisons

In order to verify the conclusions listed in the 90-day report, statistical comparisons on endpoints of interest¹⁰ have been made using the following sources in the report:

⁹ Background concentrations of Total IgE in Sprague-Dawley rats are 0.6 ug/ml (Abadie and Prouvost-Danon, 1980); this is equivalent to 250 IU/ml. It is physically impossible to measure less than one IU/ml of IgE in any rat, and even more impossible in 80 rats.

¹⁰ Endpoints of interest were selected from quick visual check of data summary tables. This is not an exhaustive analysis of all raw data from the 90-day report.

- Appendix A3: Individual animal organ weight absolute values
- Appendix A4: Individual animal organ weight relative values
- Appendix B1: Individual animal hematology
- Appendix B2: Individual animal clinical chemistry

Table 3. Results of statistical analysis of raw data from the 14 day study

Toxicological endpoint	Test group mean values females/males/total	
	Vehicle control group (G1)	Bt brinjal group (G4)
Organ weight – ovaries (g) females only	0.11	0.06**
Organ weight – spleen (g) females/males/total	0.86/1.34/1.10	1.02/1.19/1.11
Organ weight – kidneys (g) females/males/total	1.42/1.34/1.38	1.48/1.19/1.34
Total white blood cells (x10 ³ /cmm) females/males/total	9.3/11.1/10.2	14.0*/12.6/13.3*
Aspartate aminotransferase (AST) females/males/total	134.5/189.5/162.0	151.7/156.5/154.1
Plasma acetylcholinesterase (IU/L) females/males/total	591.6/604.0/597.8	875.0/902.6**/888.8**
RBC acetylcholinesterase (IU/L) females/males/total	299.9/388.3/344.1	265.7/335.6/300.6
Total acetylcholinesterase (IU/L) females/males/total	891.4/992.4/941.9	1140.7/1238.2/1189.4*
Bilirubin (mg/dl) females/males/total	.58/.51/.54	81**/.52/.66*

*Statistically significant difference from rats fed Bt brinjal at $p \leq 0.05$

**Statistically significant difference from rats fed Bt brinjal at $p \leq 0.01$

According to the 2008 study protocol, toxicological implications of the results must be reported:

“The 90-day study provides information on the possible health hazards likely to arise from repeated exposure over a prolonged period of time covering post-weaning maturation and growth well into

adulthood. The study will provide information on the major toxic effects, including possible target organs, and the possibility of cumulative effects.” –DBT, 2008

Discussion on the implications of the toxicology results for the 90-day report from INTOX has been left out.

Toxicological implications of the lab results in Table 3 from 90 day study

Females who were fed Bt brinjal had smaller ovaries than controls. At just over half the expected size, ovaries of Bt brinjal-fed rats exhibit a consistent (vegetable controls also had normal-size ovaries compared to Bt-brinjal rats, see Appendix B, Table 2) and profound reproductive toxicity signal that is statistically significant at $p < 0.0001$ ¹¹ even with the small number of animals tested and the relatively short exposure time (90 days). Unfortunately, a 90-day study is not long enough to know what the long term reproductive performance outcome would be for animals fed Bt brinjal. Other research has shown that when mice were fed genetically modified food containing the Bt toxin in a multigenerational study, they had decreased reproductive performance as demonstrated by smaller litter size and lower average litter weight (Velimirov 2008)¹².

Spleen weights among Bt brinjal female rats were 19% higher than the control group (and 26% higher than the vegetable control group – see Appendix B, Table 2). Differences were statistically significant when compared to the vegetable control group (Appendix B, Table 2)¹³. These differences were not noted in the INTOX report.

Significant changes in both the ovarian and spleen weights for the female rats fed Bt brinjal were apparent from summary values listed in Table 6 of the 90-day report. However, page 21 of the report incorrectly summarized the results, saying:

“The values of absolute and relative weights of kidneys, liver, adrenals, testes, spleen, brain and ovaries of male/female rats treated with Transgenic Bt brinjal containing cry 1 A(c) gene, non-transgenic brinjal and nontransgenic brinjal (commercially available) at 1000 mg/kg were found to be comparable to those of the control group rats at termination of the treatment.”

Page 22 of the report further incorrectly concludes:

“No alterations in the absolute and relative organ weights of rats treated at 1000 mg/kg [were found]”.

¹¹ Statistical significance remained the same using ovary weights relative to total body weight.

¹² It is worth noting that only one of two reproductive toxicology study protocols was powerful enough to observe this sensitive outcome; the Reproductive Assessment by Continuous Breeding (RACB).

¹³ Statistically significant differences are dependent on sample size: doubling the observations of each group G1 and G4 results in statistically significant increases in spleen weights for females fed Bt brinjal.

These statements clearly indicate that the authors are not familiar with the principles or procedures for evaluating their own results in the 90-day study. Differences between Bt brinjal-fed rats and appropriate controls are warranted. Only the vehicle control group (G1) is required in this study (DBT 1998, page 61)(OECD 1998, Item 14 page3). As shown in Table 3 above, significant measures of organ toxicity to female rats consuming Bt brinjal in the 90-day study are evident. Findings of statistical significance further emphasize the seriousness of these differences.

Rats fed Bt brinjal also displayed elevated white blood cell counts (up to 50 percent higher among females and 33 percent higher overall), compared to controls. This was consistent among vegetable controls (Appendix B, Table 2). In differentiated white blood cell counts, this increase is composed mainly of a near doubling in the count of eosinophils (a type of white blood cell) among Bt brinjal-fed rats. Eosinophils typically increase in response to allergic disorders, infection or to epidermal inflammations (such as those caused by parasitic infections). Bt brinjal-fed female rats had nearly twice the concentration of eosinophils compared to control groups, consistent with subchronic gut irritation, possibly caused by Cry1A(c) protein in the diet. For reasons discussed above, it is impossible to compare eosinophil concentrations using the raw data presented in the 90-day report.

Bilirubin concentrations are also elevated among female rats, with a 35% increase compared to vegetable controls (Appendix B, Table 2) and a 40% increase compared to vehicle controls. Bilirubin is a measure of liver function. Increased bilirubin in the absence of harmful xenobiotics or infectious hepatitis indicates obstruction of the biliary ducts in the liver. The increases in bilirubin of females in this study are extreme enough to result in statistically significant differences for females and the total groups. It is not clear how the authors missed this result, but the differences are not described or discussed in the 90-day report.

Plasma acetylcholinesterase (a neurotransmitter enzyme) is 20-49% higher among all Bt brinjal-fed rats, both male and female, compared to control groups. Statistical significance for these increases were observed in comparison with the vehicle control group among males and total. The implications for elevated acetylcholinesterase in plasma samples could include early onset of type II diabetes or neurological diseases (Cargia-Ayllon et al 2010).

When combining these individual signs of toxicity, a more concrete picture emerges:

- Increased white blood cell counts coincide with enlarged spleen weights observed among Bt brinjal-fed rats, further indicating immune responses to toxic exposure.
- Elevated bilirubin concentrations and elevated acetylcholinesterase concentrations are consistent with hepatotoxicity from subchronic exposure (Garcia-Ayllon et al 2006) in rats fed Bt brinjal.

DISCUSSION

Consumers, objective scientists and government representatives need to be aware of the potential health effects of new foods proposed for sale in India so they can take part in the decision about how

much risk and uncertainty they are willing to tolerate. Although animal feeding studies are limited in their representation of human responses, they form an important basis from which to gauge possible toxic response to new products. Even when these limited short-term feeding trials are conducted correctly using Good Laboratory Practices and following internationally accepted protocols, there will be some exposures that are still untested; chronic (long term) exposure to humans and animals, occupational exposure to people growing Bt-brinjal and inhalation exposure to those who process Bt brinjal.

Adverse effects of Bt brinjal exposure may be more easily transmitted by inhalation than by ingestion. As has been shown in a study with Wistar rats, inhalation exposure caused immunomodulation in control rats housed in the same room as those fed a GM Bt rice diet (Kroghsbo et al., 2008). Human reactions to the Bt toxin via inhalation have been observed in occupational settings: greenhouse workers exposed to Bt toxin in sprays developed allergic responses and elevated IgE compared to pre-exposure concentrations (Doekes et al 2004).

Previous publications from commercial seed producers on the toxicological research of transgenic foods have included multiple control groups (Hammond et al 2006). The use of multiple control groups has the effect of increasing the variation (wider confidence intervals) in the combined controls, which decreases the chance that a difference will be found between the test group and the controls. In some cases, the data are recorded under different circumstances than the data from the animals being fed the test diet. In toxicology research, comparing equal numbers of individuals from two groups that receive different diets while all other variables are kept constant is the established method for investigating health effects related to diet. Establishing dose and response effects requires *at least* three test diet dose groups. Somehow the presentations made by commercial seed producers have allowed the opposite set of comparisons to be made: one or two test doses compared to several control groups, often not conducted under the same conditions.

Extra control groups is only one technique used by commercial operators to attempt to disregard significant differences between animals fed genetically modified foods and those on conventional diets. Other “techniques” that would disqualify research results from publication (if reviewers and publishers were blinded to the author’s interests) include:

1. A false assertion that males and females must have the same toxicological responses;
2. A false assertion that, if two doses are used, the higher dose must have a greater effect than the lower dose (a so-called dose-response observation);
3. A total omission of any data analysis enabling researchers to write conclusions in the void of data evidence;
4. A total omission of statistically significant differences in organ weights, haematology and clinical chemistry; and

5. Conclusions that ignore toxicologically significant results.

The findings from this set of raw data from acute and sub-chronic exposures are consistent with previous findings of hepatic toxicity in rats fed Bt foods containing the Cry1A(c) protein (Spiroux de Vendômois 2009).

“Should there be structural alerts for reproductive/developmental effects or other indications from data available on a GM food and feed, then these tests [multi-generational reproductive toxicity studies] should be considered” – European Food Safety Authority GMO Panel Working Group on Animal Feeding Trials (2008)

Reproductive toxicity of Bt brinjal is demonstrated by the reduced ovarian weights resulting from a dose of 1000 mg/kg-day (ie 1 gram/kg body weight each day) using only ten animals compared to two groups of controls.

The likely clinical significance of decreased ovarian weights is lower fecundity, although other unintended effects may occur as well. This brings into question the possibility of hormonally-mediated toxicity that has not previously been considered for Bt brinjal but has been observed in other studies on GM foods containing the Cry1A(c) protein (Seralini et al 2007) and the Cry1A(b) protein (Velimirov et al 2008). This toxic effect was completely missed by the toxicologists who wrote the report for the 90-day study, the company reviewers who received the report, and the government committee who subsequently reviewed the report. If the statistical analysis had been conducted as indicated in the methods section of the report and the results of the analysis had been included in the summary tables and discussion as required by the regulatory guidance, this would have been impossible to miss.

The logical next steps for describing the risk profile of Bt brinjal are to:

- conduct proper dose limit testing as required by the 1998 (revised in 2008) guidelines.
- conduct the 90-day subchronic feeding study according to guidelines. That is, use at least three dose groups, include IgE measurements, perform daily observations on animals and include behavioural tests on individuals, include statistical analyses comparing the Bt brinjal group with appropriate controls and report results accordingly.
- complete multigenerational studies as suggested by the European Food Safety Authority to assess reproductive performance outcomes.

Commercial release of this product is not recommended prior to adequate safety testing. The minimum number of toxicity studies as recommended in the DBT 1998 protocol have not been conducted on Bt brinjal, but even meeting these requirements would be an improvement on current efforts.

The compositional analysis (reported in section 7.2 of Mahyco 2008) describes Bt brinjal as similar to non-Bt brinjal in content of protein, carbohydrate, oil, calories, ash, nitrogen, crude fibers and moisture content. These analyses were conducted by the seed company (Mahyco) at their own labs. Results are

not shown in this report so it is impossible to know how large these differences might have been. Was the conventional counterpart of Bt brinjal used for the compositional analyses? The conventional brinjal parent variety was not named in the Mahyco 2008 report or the toxicology study reports. Was the conventional counterpart of Bt brinjal used in the 14-day and 90-day toxicology studies? If specific differences in vitamin, mineral, fatty acid and protein contents of the brinjal and Bt brinjal diets were not known at the time of the studies, there is some uncertainty about nutritional equivalence between test groups.

In particular, we have no knowledge of whether or not the Cry1A(c) and other protein concentrations in the dried brinjal powder used in this research was representative of actual cooked fresh brinjal at the point of consumption. Storage conditions of this brinjal powder are important, as we are led to believe that both the brinjal and Bt brinjal powder were received by INTOX in a single shipment from Mahyco and fed to rats over a period of years. There is no chain of custody report or acknowledgement of sample receipt, no verification of transgenic material presence and absence upon sample receipt and no documentation of proper labeling or safe storage procedures. It is likely that pesticide concentrations in the non-Bt brinjal and the Bt brinjal were measurable both prior to drying and before feeding to rats, yet we have no data on that either.

Brinjal is an exceptional plant with many varieties. Ideally, the selection of non-Bt brinjal would have been the parent (conventional) variety of brinjal, grown in the same location at the same time as the Bt brinjal to minimize differences in nutrients and solanine content.

CONCLUSION

A review of the adequacy of current toxicology studies to address the safety of genetically modified Bt brinjal for commercial release shows that the studies were not conducted according to the published standard, did not accurately summarize results, and ignored toxic endpoints for rats fed Bt brinjal.

It is obvious to an independent reviewer that the 90-day toxicity study was conducted at the particular dose of 1000 mg/kg-day with the expectation of finding no evidence of toxicity. According to the 1998 guidance,

“If a test at one dose of at least 1000 mg/kg body weight (but expected human exposure may indicate the need for a higher dose level) using the procedures described for this study produces no observable toxic effects, then a full study using 3 dose levels may not be necessary. The treatment schedule is given below: G1= control group, G2= Nontransgenic vegetable group, G3=Transgenic vegetable.”

Were the contract laboratory INTOX PVT LTD and the funder Mahyco uncomfortable with results showing evident toxicity among rats fed Bt-brinjal? Did the researchers write the conclusions for the 14-day and 90-day studies themselves or did others write conclusions for them? These questions are of

interest since the text does not match the data, the researchers did not sign their reports, and the cover page of the 90-day report details a completely new report number (R/2183/SOR-90) from that which may be the original, 05.0002.

Not only has the scrutiny of these data provided insight into the substandard and extremely misleading interpretation of results, but it suggests to the reviewer that urgent changes need to be made to ensure that future studies are properly conducted.

In particular, current results from these rat feeding studies indicate that rats eating Bt brinjal experienced organ and system damage: ovaries at half their normal weight, enlarged spleens with white blood cell counts at 35 to 40 percent higher than normal (elevated eosinophils in particular) indicating immune function changes possibly due to allergen response, and toxic effects to the liver as demonstrated by elevated bilirubin along with plasma acetylcholinesterase. Behavioral effects, neurological function, reproductive performance, and reduced resilience in circumstances of infection or other adverse events have not been addressed in these studies.

Unanswered Concerns regarding the safety assessment of Bt Brinjal:

Nutritional and Toxicological Equivalence of dried Bt brinjal samples

Are dried brinjal samples equivalent to cooked brinjal as it is prepared for human consumption, or do dried samples differ in their concentrations of Cry1A(c) and other important proteins, carbohydrate, fat and micronutrients? Data on heat stability of Bt brinjal shows a decline in Cry1A(c) concentrations under laboratory conditions (Mahyco 2008), so how much of this new protein was actually in the dried samples? Would this concentration be the same as that in brinjal in cooked human food? Would the toxicity profile of Bt brinjal also change as a result of cooking and home processing? Notably, Codex recommends:

“The potential effects of food processing, including home preparation, on foods derived from recombinant-DNA plants should also be considered. For example, alterations could occur in the heat stability of an endogenous toxicant or the bioavailability of an important nutrient after processing. Information should therefore be provided describing the processing conditions used in the production of a food ingredient from the plant. For example, in the case of vegetable oil, information should be provided on the extraction process and any subsequent refining steps.” paragraph 47

Dietary equivalence for brinjal-fed rats, Bt brinjal-fed rats and vehicle control rats has not been addressed.

Inhalation exposure to Bt brinjal

Oral ingestion of Bt brinjal does not address the issue of inhalation exposures to people who grow Bt brinjal or live near Bt brinjal crops in the ground. Toxicological responses to proteins that reach the lining of the lungs and nasal cavity, previously found to be of concern for agricultural workers, have not been addressed.

Toxicity testing standards

The main reason for conducting the toxicology studies is to have an objective assessment of whether or not the new food is safe for humans to eat. This needs to be a careful and objective assessment since millions of people with varying nutritional status, age and biological resilience will be exposed in the event of commercial release.

Neither of the 90-day toxicity testing protocols released by the Department of Biotechnology (1998 and 2008) are as methodologically strong as accepted international standards (see Appendix 1). This makes India an “easy target” for Mahyco company since the requirements to conduct toxicology studies are less stringent those in the European Union.

The use of laboratory animals to test food safety for humans is already a significant departure from species-specific testing. Deviations and omissions from accepted protocols need to be checked. Yet every departure made by INTOX on behalf of Mahyco has resulted in lower standards with less power to detect changes in rats eating Bt brinjal. These include leaving out important endpoints such as IgE measurement to test for allergenicity, using only one dose group that is smaller than human consumption is likely to be, ignorance of toxicological equivalence, lost data, lack of Good Laboratory Practices standards, inadequate observations of animals, a 29% decrease in exposure days (doses were administered 5 days per week instead of 7), failure to quantify Cry1A(c) concentrations in dried fruit powder, etc.

The real risk here is that potential health problems attributable to Bt brinjal will be ignored as masses of people eat the very food their government thought was safe.

Adoption of and adherence to a stronger safety testing protocol in India that is scientifically based is prudent, given the large buffer zone of trust bestowed on commercial interests in the case of Bt brinjal.

In the long run, it is the people of India who will pay the price for bad science!

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Appendix A: Protocol Requirements for 90-day toxicity study by various sources

	DBT 1998 ¹⁴	DBT 2008 ¹⁵	OECD 1998 ¹⁶	FDA Redbook 2003 ¹⁷
Maximum number of animals per cage	Not specified	Individually or in groups of no more than 5	Individually or in small groups of the same sex	Individually
Good Laboratory Practices	15 items	Not specified	Not specified	U.S. FDA good laboratory practice (GLP) regulations, issued under Part 58. Title 21. Code of Federal Regulations
Number of dose groups	At least 3	At least 3	At least 3	At least 3 but ideally 4 or 5
Nutritionally equivalent diets required for each group	No	No	No	Yes
Number of animals per dose group	20	20	20	40 (20 if longer-term studies are planned)
Age of animals	Six to eight weeks	Healthy young	As soon as possible	No later than six

¹⁴ Department of Biotechnology (1998) Revised Guidelines for Research in Transgenic Plants and Guidelines for Toxicity and Allergenicity Evaluation of Transgenic Seeds and Plant Parts. Ministry of Science and Technology, Government of India. Public Printing Service (Delhi) 96 pp.

¹⁵ Department of Biotechnology (2008) Protocols for Food and Feed Safety Assessment of GE Crops. Ministry of Science and Technology, Government of India. 38 pp.

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	old	adult animals	after weaning, before they are 9 weeks old	to eight weeks old
Method of administration	Dry powder added to peanut oil and administered by gavage. Oil volume not to exceed 1 ml/100 g body weight	Not specified	Gavage delivery of an aqueous solution/suspension or solution/emulsion in corn oil. Oil volume not to exceed 1 ml/100 g body weight	In the diet, dissolved in drinking water or by gavage. Oil gavage not to exceed 0.4 ml/100g body weight
Control groups required	Vehicle control	Conventional non-GM plant with similar nutritional values	Vehicle control	Vehicle control: Control diet is equivalent in caloric density and contains the same levels of nutrients (e.g., fiber, micronutrients) as the diets of the test groups
Dosing regime	5 days per week	7 days per week	7 days per week	7 days per week
Observation of animals	Daily observations of tremor, convulsion, diarrhoea, lethargy, dyspnea and nasal bleeding	Clinical signs include, but are not limited to: rapid weight loss; diarrhea (if debilitating); progressive dermatitis; rough hair coat; hunched posture; lethargy or persistent recumbency; coughing; labored breathing; nasal discharge; jaundice or anemia; neurological signs; bleeding from any orifice; self-induced trauma; any condition	Clinical observations at least once per day after dosing. Twice daily observations of morbidity and mortality. Ophthalmological exams at beginning and end of trial Behavioural tests: sensory reactivity to stimuli of different types (e.g., auditory, visual and proprioceptive stimuli), assessment of grip strength and motor activity assessment.	Daily or twice daily. Observation of general pharmacologic and toxicologic effects but also of neurologic disorders, behavioral changes, autonomic dysfunctions, and other signs of nervous system toxicity including but not limited to changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions or

		interfering with eating or drinking (e.g., difficulty moving); or excessive or prolonged hyperthermia or hypothermia		other evidence of autonomic activity (e.g., lacrimation, piloerection, pupil size, unusual respiratory pattern). ¹⁸
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¹⁸ Additionally, changes in gait, posture and response to handling, as well as the presence of clonic or tonic seizures, stereotypes (e.g., excessive grooming, repetitive circling) or bizarre behavior (e.g., self-mutilating, walking backwards) should be recorded. Tumor development, particularly in long-term studies, should be followed: the time of onset, location, dimensions, appearance and progression of each grossly visible or palpable tumor should be recorded.

Out-of-the-cage behavioural tests are conducted prior to treatment start and periodically throughout the study.

Appendix B: New Statistical Analyses of Bt-brinjal-fed rats in 14-day and 90-day feeding trials

Table 1. Results of statistical analysis of raw data from the 14 day study

	Arithmetic mean values for females/males/total		
	Vehicle control group (G I)	Vegetable control group (G II)	Bt brinjal group (G IV)
Total white blood cells ($\times 10^3$ /cmm) females/males/total	8.6/9.0/8.8	8.7/8.4/8.6	7.7/8.2/8.0
Aspartate aminotransferase (IU/L) females/males/total	164.2**/154.0*/159.1**	165.4*/149.8*/157.6**	251.8/244.8/248.3
Plasma acetylcholinesterase (IU/L) females/males/total	641.8/656.2/649.0**	557.7/621.5/589.6	534.0/529.3/531.7
Red blood cell acetylcholinesterase (IU/L) females/males/total	407.6/398.8/403.2	303.0/369.7/336.4	351.9/324.9/338.4
Bilirubin (mg/dl) females/males/total	1.1/0.9*/1.0*	1.1/1.1/1.1	1.1/1.2/1.2

*Statistically significant difference from G IV at $p \leq 0.05$

**Statistically significant difference from G IV at $p \leq 0.01$

Table 2. Results of statistical analysis of raw data from the 14 day study

	Test group mean values females/males/total		
	Vehicle control group (G1)	Vegetable control group (G2)	Bt brinjal group (G4)
Organ weight – ovaries (g) females only	0.11**	0.10**	0.06
Organ weight – spleen (g) females/males/total	0.86/1.34/1.10	0.81*/1.20/1.00	1.02/1.19/1.11
Organ weight – kidneys (g) females/males/total	1.42/1.34/1.38	1.49/1.20/1.34	1.48/1.19/1.34
Total white blood cells ($\times 10^3$ /cmm) females/males/total	9.3*/11.1/10.2*	9.3*/10.3/9.8*	14.0/12.6/13.3
Aspartate aminotransferase (AST) females/males/total	134.5/189.5/162.0	152.7/166.0/159.4	151.7/156.5/154.1
Plasma acetylcholinesterase (IU/L) females/males/total	591.6/604.0**/597.8**	731.0/753.2/742.1	875.0/902.6/888.8
RBC acetylcholinesterase (IU/L) females/males/total	299.9/388.3/344.1	332.1/390.1/361.1	265.7/335.6/300.6
Total acetylcholinesterase (IU/L) females/males/total	891.4/992.4/941.9*	1063.1/1143.3/1103.2	1140.7/1238.2/1189.4
Bilirubin (mg/dl) females/males/total	.58**/.51/.54*	.60**/.52/.56**	.81/.52/.66

*Statistically significant difference from rats fed Bt brinjal at $p \leq 0.05$

**Statistically significant difference from rats fed Bt brinjal at $p \leq 0.01$