Technical Opinion

The President of the National Technical Commission on Biosafety - CTNBio, in exercise of its powers and in accordance with Article 14, paragraph XIX of Law 11.105/05 and Article 5, paragraph XIX of Decree 5.591/05, announces that the 161 Ordinary Meeting, held on April 18, 2013, CTNBio appreciated ratified the position of the presidency on the following topic:

CONSIDERED OPINION

The President of the Brazilian National Technical Commission on Biosafety - CTNBio, in response to the demand of the Ministry of Foreign Affairs, appointed a committee comprised of four distinguished researchers who evaluated the work of Séralini and his collaborators in prior publication on the journal Food and Chemical Toxicology, available on the website http://ac.els-cdn.com/S0278691512005637/1-s2.0-S0278691512005637-main.pdf?_tid=bdde0922-2296-11e2-ada7-00000aab0f6c&acdnat=1351604340_c8d8f6b6fbeeec91e0ef4b1ca2444c8f . The result of this evaluation is below. This Technical report was issued by the President of CTNBio on October 24, 2012 and confirmed in 161 regular meeting of the commission.

Document evaluated

Séralini GE, Clair E, Mesnage R, Gress S, Defarge N, Malatesta M, Hennequin D, de Vendômois JS. Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. Food Chem Toxicol. 2012 Sep 11. pii: S0278-6915(12)00563-7. doi: 10.1016/j.fct.2012.08.005

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Summary

In an overall assessment, this study represents a strong commitment to assess the consequences of a diet with genetically modified (GM) plants, exposed or not to the herbicide to which they are resistant, as well as with the herbicide itself, to rats after long-term treatment. Results generated could potentially bring valuable informationabout the issue raised by the authors, however, the study completely fails to reach suchpurposes, due the following main reasons:
(1) The rat strain was poorly selected for the study, inasmuch as it is known thatSprague-Dawley rats develop tumors spontaneously in greater frequency than

otherstrain (Keenam et al., 1979). The choice of another strain would have given greaterconsistency and reliability to the set of results of the study.

(2) Results are dramatically described and illustrated in a non-conventional fashion,leading to a relationship between feeding on GM plants and higher mortality or appearance of tumors, without, however, presenting numerical data in most analyzes, or statistical analyzes that inform the level of significance of the data presented in ageneral way. A study of the document shows that the expression "statistic" appears only
twice: in item 2.6, "Statistical analysis" in Materials and Methods; and in item 3.3 (Results, biochemical analyses), in the phrase "For biochemical measurements in rats,statistical analysis". This confirms that data presented as regards biochemical parameters were the only results submitted to statistical analysis, even so, rather unclear.

(3) Other points recommend caution with respect to the study, such as: Lack of a definition of the control maize lineage, described only as "closest isogenic maize" (Table 1); similar results are observed with GM maize treated or not with the herbicide and with the herbicide itself, without dose-effect relation; the number of animals per group is very small (10 males and 10 females), particularly considering specific sex differences;for several of the results, groups "treated" (n=90) are compared with "controls" (n=10)for each sex, while control and experimental groups should have similar sample size.

Finally, the review of the study indicates that based on the results presented it is not possible to establish any conclusions about the long-term effect of feeding on GM maize, treated or not with the respective herbicide, in rats. To this end, the findingsshould be described more accurately and submitted to a consistent statisticalevaluation.

Therefore, this opinion indicates the main technical limitations, which invalidate the findings presented by the authors.

1 Introduction

This paper consists of a critical evaluation of the scientific publication "Séralini GE, Clair E, Mesnage R, Gress S, Defarge N, Malatesta M, Hennequin D, de Vendômois JS. Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. Food Chem Toxicol. 2012 Sep 11. pii: S0278-6915(12)00563-7. doi: 10.1016/j.fct.2012.08.005". The evaluation was performed following the standards observed to review scientific articles, being solely based on information disclosed in the article.

The main purpose of this study was to investigate the long term effect of a genetically modified (GM) maize diet, treated or not with the respective herbicide, as well as the effect of the herbicide itself, in rats. An analysis of the context in which this publication is included shows that the issue is highly relevant, for it is an area of research in whichseveral other groups have conducted studies with similar goals. Recently,

Domingo and Giné Bordonaba (2011) and Snell et al. (2012) published reviews including several studies that assessed the safety of food with GM plants.

In the review of Domingo and Giné Bordonaba (2011), they point out the main results of 27 studies. Of these, only 4 indicate possible changes in animals fed on GM plants when compared to the controls. Two of these studies are of Séralini's group, and two of the group of Italian researcher Manuela Malatesta. The review of Snell et al. (2012), more stringent for considering only long-term experiments, stresses among 24 studies only 4 indicating harmful effects of feeding GM plants. All belong to Malatesta's group (coauthor of the study evaluated here). Thus, one can observe a trend of smaller groups, among which fits that of Séralini, in obtaining results indicating a deleterious effect of GM plants on in vitro and in vivo studies.

The article calls attention because of the strong visual appeal of images of rats bearing large tumors, associated with feeding on a diet containing GM maize. The scientific, political and economic impact of the results reported in this publication requires its analysis in more details. Accordingly, it will be pointed out various aspects regarding the methodology applied and conclusions.

2 Methodology

For the assessment of long-term toxicity, male and female Sprague-Dawley rats were used, fed for 2 years a genetically modified (GM) and Roundup (R) tolerant (NK603) maize diet, grown or not with the herbicide (3 L ha-1; 540 g/l glyphosate). The standard diet (AO4; Safe, France) was prepared to contain dry 11, 22 or 33% GM maize cultivated or not with R, or 33% of the isogenic non-transgenic maize (control diet). The concentrations of transgenics were confirmed in each diet by qPCR. Similarly, residues of R were determined on tissues by mass spectrometry.

The protocol of the study involved 20 experimental groups, each with 10 animals. Twelve groups consisting of male rats (6 groups) or female (6 groups) ate each of them diets prepared with different increasing concentrations of NK603, cultivated or not with R (11, 22 or 33% of maize), respectively. Two other groups, one of males and another of females ate the control diet for the same period (33% maize). Finally, six other experimental groups (with 3 with males and 3 with females) also received the control diet, but had to ingest in their drinking troughs, each, water supplemented with 1.1 x 10-8%, 0.09% or 0.5% R, respectively.

During these 2 years of the study, 11 blood samples were obtained from animals, under anesthesia with isoflurane, to perform 31 biochemical analyzes. Likewise, 11 24-hour urine samples were collected, which were quantified as to 16 parameters. At the time of euthanasia of animals by exsanguination under anesthesia with isoflurane, liver samples were also collected to perform three different biochemical analyses. Moreover, samples from 36 different tissues were also obtained, including of neoplasms, for anatomic pathology evaluation under H&E staining. Samples of kidneys, livers and neoplasms were subjected to transmission electron microscopy. When necessary, due to ethical issues (such as loss of 25% of body weight, presence of neoplasms with more

than 25% of body weight, occurrence of bleeding or prostration), rats were euthanized during the study. It was basically used descriptive statistics with discriminant analysis.

A more detailed assessment of the materials and methods used in the study shows the existence of serious limitations, as described below:

2.1 Experimental animals:
The laboratory rats used were of Sprague-Dawley strain of both sexes and five weeks old at the beginning of the experiment (young adults).

Sprague-Dawley strain has been used since the 70's as a great model to study toxicity of various substances, including allowing the induction of tumors for the development of biological models for studies of anticancer substance. However, its use in long-term studies, particularly those in which the development of tumors is used as a parameter, does not seem suitable. Such question is based on the fact that this strain, as well as other laboratory rats, naturally presents high incidence of tumors as they reach menopause (450-540 days of age) and, subsequently, senility (600-800 days of life).

The frequency of spontaneous mammary tumors in rats varies according to the strain, however, being lower in animals strains such as Wistar, Long-Evans, Noble, WAG/Rij and BN/BiRij (Walsh and Poteracki, 1994; Poteracki and Walsh, 1998; Sommer, 1997; Cheung et al. 2003; Solleveld et al. 1986) than in Sprague-Dawley strain, in which the frequency of these tumors is higher (Solleveld et al., 1986; Hotchkiss, 1995; Durbin et al., 1966; Kaspareitt and Rittinghausen, 1999).

Although they present quite different metabolisms, with regard to tumor development, male rats also show increased incidence of tumors at advanced age. Drori and Folman (1976) point to the natural occurrence of tumors in 10.4% of male rats analyzed. Actually, data of the study of Séralini et al. confirm the historical and already recognized observation of high incidence of tumors in strains of laboratory mice, as well as the higher incidence in females compared to males in that strain (Figures 1 and 2), according to data available in scientific literature.

Based on the facts stated above, the article under analysis makes a major mistake by proposing experimental design for the assessment of incidence of tumors in the Sprague-Dawley rat strain, at ages and physiological states and in body organs unsuitable for formulating hypotheses and conclusions about the carcinogenic effects of the substances tested.

Moreover, the use of Sprague-Dawley strain may lead to distorted conclusions as to data collected of animals at older age a regards the occurrence of tumors. Hence, the suggestion of the authors of higher incidence of neoplasm in treated animals compared to the controls (and on which is grounded the focus of discussions of the article at hand) may result from a natural incidence of tumors in menopausal and/or senile animals, for instance. Therefore, data collected from the 180-200 days of age now have low representation in the monitoring of the effects of the tested substances.

Likewise, considering the organs analyzed: hepato-digestive tract, kidneys, mammary gland and pituitary, given the characteristics it is extremely difficult, if not impossible, to distinguish the cause of the occurrence of such tumors in experimental groups such as those analyzed in this paper. It should be noted that these tumors tend to naturally occur in senile stage or are hormone-dependent influenced by ultradian hormonal cycles – as, for instance, puberty and menopause

2.2 Number of experimental animals

Given the limitations pointed out in relation to animal species used in this study (strain, age and organs), the use of 200 animals, divided into 10 experimental groups of each sex, caused statistical analyzes to be implemented in the experimental groups with only 10 animals per treatment/sex. Even if such number is accepted and recommended for toxicity studies in rats, including as noted in Table 1 of the article, which mentions a previous study (Hammond et al. 2004) and regulatory frameworks, the existence of several factors that may impair the analysis of parameters assessed, the sample size should be substantially expanded.

The use of 10 animals per experimental group is recommended, in case of toxicity studies in rats, when using young adult animals, that is, outside the ages/physiological moments where the incidence of tumors is naturally very high. In this case, when observed changes in the incidence of tumors in a study designed that way, further investigation to assess possible negative effects of the tested substances to the health of the animal should be conducted.

The study under examination intended to go beyond what regulatory frameworks and previous studies advocate, by proposing an extension of time tracking and increased number of analyzes performed in animals undergoing treatment with GM maize NK603 and agrochemical Roundup. However, the authors not only make mistakes in the choice of the animal model (Sprague-Dawley rat strain), as described above, but persist in the mistake by not proposing alternatives to overcome the effects arising from the use of this rat strain, such as, higher number of experimental animals (treatment and control groups) and the use of castrated animals, which could possibly reduce the effect of hormonal interferences in the analyses.

2.3 Statistical methods

The main questions regarding the statistical analysis of data of this study are, having analyzed continuous variables, why was not variance analysis used among groups? In this case, one would be able to identify significant differences among groups for each of the variables evaluated. Such information is not clear in the results presented in the article. It should be pointed out this can easily confuse readers as to the true significance of possible differences mentioned by the authors.

Hence, to the detriment of the classical statistical and conventional methods for experiments along the lines of those performed in this study, the authors used several multivariate analyzes, justifying their "...robustness in modeling, analysis and interpretation of complex biological and chemical data... (sic)." This type of statistic, which involves methods such as Principal Component Analysis (PCA) and Least

Squares in samples with Structure (OPLS and PLS), is, in fact, widely used in complex biological datasets, containing, for instance, thousands of pieces of information and/or samples.

These methods are used to group data associated/correlated, allowing inferences about the response to a given factor. In cases involving bioinformatics, for example, such methods are often used to study associations of portions of the genome with a particular phenotype in a population, thereby creating hypotheses to be subsequently investigated.

In the case of the work at hand, what is presented does not quite represent "... complex biological data ... (sic)" as described by authors, given the small number of samples and analyzes made. The use of conventional statistical methods (appropriate to the study at hand), such as the Analysis of Variance (ANOVA), would probably reveal the insignificance of the numerical differences observed. For that reason, we believe the decision to apply the statistical methods proposed without prior presentation of results from standard statistical analyzes (ANOVA) is wrong. The latter should act as a first filter of the relevance of the observations.

Other limitations of the methodology:
(a) In section 2.2, it is reported that all feed consisted of balanced diets with equivalente chemical composition except for the GM and without contamination by agrochemicals. However, there was no report of how often these controls were performed, if diets were prepared from the same maize batches, or how often, and how they were stored. In this sense, and as this is a paper on the impact of chronic ingestion of diets, measures of potential concentrations of aflatoxin(s), for instance, should have been considered. Furthermore, a table with the centesimal composition of the diets should have been presented in the study.

(b) In item 2.3, authors mention that the animals were monitored twice a week, including with regard to their individual body weights and feed and water consumption. Nonetheless, such data were not presented (although the authors mention that there were no differences in feed intake and body weight; see p. 4), which leaves much to be desired, especially in this type of research, as it prevents readers from reaching the conclusion as to whether there is a need or not in the study of additional "pair-feeding" groups for the control, for instance, of the occurrence of any changes in the animals' diets. Admittedly, changes as to higher or lower calorie intake (and consequent change in body weight) for example, influence the occurrence of neoplasms. Did the animals' intake of water-supplemented Roundup not change, perhaps, the feed intake of such animals and their body weights?

(c) The proportion of GM maize or not in the feed is contrary to international nutrition standards of laboratory animals, in which only one test substance can be administer to a maximum of 10% of the diet, under the risk of altering its balance. In fact, 11, 22 and 33% of test substance are unacceptable concentrations for a rodent's diet, even if the diet simply consists of corn. Still, regarding the proportion of test items in the feed, FDA

(FDA Redbook 2000) recommends the adoption of procedures that are not covered in this paper: “When the test substance has no caloric value and constitutes a substantial amount of the diet (e.g., more than 5%), both caloric and nutrient densities of the high dose diet would be diluted in comparison to the diets of the other groups. As a consequence, some high dose animals may receive higher test article doses than expected because animals fed such diluted diets ad libitum may eat more than animals in other dosed groups to compensate for the differences in energy and nutrient content of the high dose diets. Such circumstances make it especially important that feed consumption of these animals be as closely and accurately monitored as possible in order to determine whether changes observed could be due to overt toxicity of the test substance or to a dietary imbalance. To further aid in this assessment, two control groups can be used; one group would be fed the undiluted control diet and a second group would be fed the control diet supplemented with an inert filler (e.g., methylcellulose) at a percentage equal to the highest percentage of the test substance in the diet.”

This analysis of the materials and methods used in this study shows that for the paper to try to support the conclusions suggested (that the substances tested - GM maize and Roundup, would cause damage to the health of animals) it would be necessary to sequentially and incrementally implement all actions below, described as far as the

Materials and Methods are concerned:
a. Use of a strain of trial animals that would not present high rates of tumor incidence, as it occurs in the Sprague-Dawley rat strain, followed by:
b. Use of castrated animals to mitigate the natural hormone-dependent effects in the increased incidence of tumors, followed by:
c. Minimum tenfold increase in the number of trial animals in each tested group to permit efficient statistical analysis, followed by:
d. Use of traditional statistical methods (ANOVA) to accept or reject the hypothesis that the substances tested cause negative effects to animal health, before any additional statistical speculation.

After performing all these steps, if any deviation from the expected were observed, with statistical significance, one could contemplate pertinent conclusions. Without this approach, the data presented in the study are nonconclusive and do not allow for the formulation of new research hypotheses, without changing the status quo of knowledge in this sector.

3 Results

The description of the results has several serious limitations that prevent a correct evaluation of the data:

3.1 Mortality

The descriptions on mortality data refer to Figures 1 and 2. Through them, the authors intend to demonstrate differences in mortality rates between the groups, which are discussed in the text in section “3.1 Mortality”.

In addition to the criticisms in the previous section (as far as the choice of animal species, experimental design and statistical analysis are concerned), which in themselves have disqualified the study, we see that they use expressions such as “times” or “fold” whenever they want to express the magnitude of the difference between what was observed in a treated group when compared to the control group. It would be reasonable to expect that the authors would present the significance level of the statistical tests applied to the data collected, by simply stating whether the groups differed or not, something impossible given the lack of appropriate statistical analysis (ANOVA). For example, one of the most striking sentences in the article summary says that “all treated groups died 2–3 times more than controls, and more rapidly” - however, no statistical analysis indicate whether this difference is significant or not with the numbers of animals used (20 in the control group against 180 in the treated groups). Furthermore, figure 1 itself is very illustrative but presents very little information – there is no average, standard deviation, or other information that provide consistency to the meaning of the results of an experiment.

Therefore, given the wrong choice of the statistical analysis strategy, any argument that may arise from the above becomes useless. As a consequence, any conclusions on animal health about the negative effects of the substances tested (measured by the mortality rate) are meaningless, because what was observed and the way it was analyzed, prevent us from doing so.

3.2 Anatomopathological comments

The description of the anatomopathologic results (item 3.2) does not comply in several respects with the conventional standards for scientific publications. In the second sentence of this item, the authors say “All data cannot be shown in one report, and the most relevant are described here.” Choosing what is "most relevant" can be very subjective, thus providing unreliable information. The analysis and the terminology used as far as the "tumors" are concerned is rather incomplete. Thus, at no time are terms used and parameters evaluated, such as “latency”, “incidence” or “plurality” of neoplasias, as it would be necessary in the text and in Table 2. Moreover, once again, the authors exceed in the use of “times” and “fold” to express the magnitude of the alleged differences between what was observed among the trial groups, at the expense of the direct response of whether or not there was a significant difference among the treatments.

Despite the importance given to the appearance of tumors, the article does not report the frequency with which these tumors are found in treated or control animals. The text (page 4, paragraph 4) uses vague descriptions, such as “10–30% of treated females per group developed tumors”, “50–80% of female animals had developed tumors in all treated groups” or “70–80% of animals presented 1.4–2.4 times more abnormalities than controls”. Table 2, which provides the figures, also vaguely refers to the “most frequent anatomical pathologies observed”, even without indicating the total number of animals per group (which must be varied, as deaths occurred throughout the study – which number in each group is not shown either). Tumors are broadly illustrated in Figure 3, but again, no precise figures are presented on their frequency in treated or control animals, or statistical significance of the numbers of tumors found.

Therefore, apart from what has been stated above regarding design errors, for example, as it does not consider the confusing effects inasmuch as the incidence of tumors or prevent conventional statistics (ANOVA), the authors provide the reader with a simple anatomopathologic survey of the occurrence of tumors in rats, describing types, affected organs and other descriptions, as the treatments did not show any deviation from the expected (proving the safety of the tested substances).

3.3 Biochemical analyzes

The claim that the biochemical analyzes also suggest pathological changes have occurred due to the treatment with the tested substances (GM maize and Roundup) is based on questionable and imprecise statistical results.. For example, the first sentence on page 6 states that “In addition, cytochrome activities also generally increased in the presence of R (Roundup)…” The word “generally” would be appropriate if it were describing a table with presentation of figures, which however does not exist; this is the only description of the results. Other interpretation errors can be identified. The statement made at the end of page 6, “The GM maize fed groups either with or without R application (in plants) showed a reduced transcription in mRNA and rRNA because of higher heterochromatin content, and decreased nucleolar dense fibrillar components.”, for example, is groundless. A cytological evidence such as "higher heterochromatin content" or "decreased nucleolar components," mostly without any quantitative evaluation, does not justify any conclusions on the level of transcription of ribosomal or messenger RNA.

Mentioning the disrupting endocrine effects also seems to be reckless and devoid of rationale based on the observed results. The evaluation of interference effects with the endocrine system can be determined by the OECD 407 test - Report of the validation of the updated test guideline 407: repeat dose 28-day oral toxicity study in laboratory rats.

No results presented seem to correlate with changes in the endocrine glands due to the administration of genetically modified maize to Sprague Dawley rats.

Therefore, if data on mortality and incidence of anatomopathologic lesions (tumors) lacked solid grounds to justify the toxic effects of the tested substances, the data for serum biochemistry follow the same line by identifying, by using unconventional statistical methods , changes in the renal and endocrine system, linking them as the effect of the existing tumors in some animals.

In conclusion, the evaluation of the results demonstrates a tendency only towards the exposure of what would favor the theory that the tested substances (maize NK603 and Roundup) would present toxic effects on the animals' health. Basic statistics on mortality data (ANOVA) is not presented, figures that do not contribute towards the elucidation of the facts are exploited as if they were new scientific results and the analysis of the changes in the biochemical profiles is questionable, since it advocates the thesis that these are caused by tumors, which in turn are inherent to the growth of the Sprague-Dawley strain.

4 References

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