ACCEDIT

CRITICISMS AND IMPROVEMENTS OF STRATEGIES FOR THE SAFETY ASSESSMENT OF GM PLANT DERIVED FOODS OR FEED

An answer to EFSA Draft report on Animal feeding trials with GMOs

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1. Overview of the safety studies of GMOs performed on mammals

Our experience in scientific committees for the assessment of environmental and health risks of GMOs, and in biological, biostatistical research and medicine, allowed us to review and criticize mammalian feeding trials with GMOs, and make new proposals. Mammalian feeding trials have been usually performed for regulatory purposes, in order to obtain authorisation or commercialisation for GM plant derived foods or feed. They may have been published in the scientific literature afterwards. We have obtained, following Court actions or official requests, the raw data of several safety, 28 day or 90 day long, *in vivo* tests on rats. We have thoroughly reviewed these tests from both a biological and biostatistical point of view.

Firstly we address the longest safety tests (which are about 90 day long) and these often analyse the biochemical blood and urine parameters of mammals eating GMOs, together with numerous organ weights and histopathology. We have focused our study on commercialized GMOs which have been cultivated in significant amounts throughout the world since 1994 (Table I). Although no detailed blood analysis is available for it, the mouse case is special because it represents the longest feeding trial available. The work has been performed by researchers independent from the GMO industry. We observe and emphasise that all the events in Table 1 correspond to soybean and maize which constitute more than 80% of the commercialised GMOs, whilst the remainder are canola or cotton (Clive, 2005, isaaa.org).

Year of reference	Plant	Pesticide contained	Name of event	Species fed	Duration	Main observations
2002[1,2] 2003[3] 2004[4] 2005[5]	Soybean	Herbicide Roundup	CP4 EPSPS	Mouse	240d	Histochemistry disturbed in several organs
2004[6]	Soybean	Herbicide Roundup	CP4 EPSPS	Rat	91d	Weight problems
2004[7,8]	Maize	Herbicide Roundup	NK603	Rat	90d	Controversial results
2006[9,10]	Maize	Insecticide mCry1Ab	MON810	Rat	90d	Significant results
2006[10,11] 2007[12]	Maize	Insecticide mCry3Bb1	MON863	Rat	90d	Controversial results

Table 1. Longest toxicity studies in mammals fed with significantly cultivated commercialized GMOs. According to the EFSA draft report on animal feeding trials for public consultation, pp 19-22, the scientific literature, and CRIIGEN data (www.crii-gen.org). We have also selected the authorized events for food and feed at least in European Union and America.

First of all, the data indicating "no problems" have been published mostly by companies from 2004, long after 10 years of commercialization in the world of comparable GMOs. This is a matter of grave concern. All GMOs have been modified to contain pesticides, either by herbicide tolerance or insecticide production. These GMOs encode only for these two traits in spite of the advertising for numerous other characters possibly existing. Usually, pesticides are tested over a period of 2 years on a mammal. Additionally, the tests last only 90 to 91 days on rats for authorized GMOs (Table 1); this duration can be considered to be very short. Moreover, they are not yet obligatory for all GMOs. The longest 240 day tests have been obtained on mice by independent researchers from 2002, studying overall histopathology at the ultrastructural level; and the latter tests have not been used to obtain the commercial release by the firm. Then 3 (and probably 4) of the 5 tests presented here show controversial results which can be discussed. Two GMOs affect the body weight increase according to the authors (RR CP4 EPSPS and MON863) (6, 12), a parameter considered as a very good predictor of other organs problems. But overall, as preliminary examples, several convergent factors appear to indicate liver and kidney effects as endpoints of GMO diet effects in these experiments.

For the longest tests performed, GM soybean available on the market was used to feed mice.This provoked the development of irregular hepatocyte nuclei, more nuclear pores, numerous small fibrillar centers and abundant dense fibrillar component, indicating increased metabolic rate (1). It was hypothesized that the herbicide residues, that this GM plant can absorb and to which it has been rendered tolerant, may be involved in these pathological features. The reversibility may be explained by the heterogenity of the herbicide residues in the feed. Anyway these are specific parameters of ultrastructural dysfunction, and the relevance is clear. The liver is reacting. It is the same in the MON810 studies where a significantly lower albumin/globulin ratio indicates a change in hepatic metabolism of 33%GM-fed rats, according to EFSA (10); taken together the results indicate potential adverse effects at this level. The insecticide produced by MON 810 could induce liver

reactions, like many other other xenobiotics. Comparable data and kidney dysfunctions are observed with MON863; we quote the initial EFSA report (and 10): « Individual kidney weights of male rats fed with the 33% MON 863 diet were statistically significantly lower compared to those of animals on control diets » ; « a statistically significant lower incidence of mineralized kidney tubules was noted for rats fed 33% MON 863 maize compared to those fed the control maize ». Even if the first effects were not over those observed with non isogenic maizes (called reference lines) containing different salts, lipids, or sugars, and even if both results are different between males and females, which is quite usual in liver or kidney pesticide reactions, they can be considered as treatment-related.

It should be noted that liver and kidney are two major organs of detoxification involved in pesticide metabolism, and that all the GMOs concerned here, like 99% of cultivated commercialized GMOs, as we have emphasised, are the so-called "pesticide-plants" designed to contain pesticides that they tolerate and/or produce (Roundup soybean and Bt maizes).

The Roundup tolerant soybean in comparison to control plants will certainly contain more glyphosate, AMPA and Roundup adjuvants, and all corresponding metabolites, as suggested by the increase in maximal levels of residues authorized in North America in Roundup-treated GM plants (EPA). These have been shown to be toxic for human placental (13) and embryonic cells (14). Roundup even stabilizes glyphosate and allows its penetration into cells, which in turn inhibits in these studies estrogen synthesis by cytochrome P450 aromatase inhibition; this changes the androgen/estrogen ratio and may explain differential impacts in both sexes. This phenomenon may thus explain at least part of the in vivo observations. All these new metabolites in edible Roundup-ready GMOs have not been assessed for their chronic toxicity and this is a major oversight in the actual regulation.

Only chronic toxicity tests (with males, females, pregnant females, and then developing progeny) that we call here the Toxotest approach or Risk management test, could address this problem. These studies should be complementary to the Safotest and the sentinel test proposed by EFSA. The Toxotest could provide evidence of potential carcinogenic, developmental, hormonal, neural or reproductive dysfunctions, as it is true for pesticides or drugs. Additionally, the 90 day long trials on adults may not scientifically replace the sensitivity of developmental tests on neonates. A good example is the gene imprinting by drugs that will be revealed only at maturity; this is an important subject of current research.

Similarly, the insecticide toxins in maize lines may have new pleiotropic or specific actions, and they can generate particular metabolites, either in the GM plant or in the animals fed with it. The Bt toxins in GMOs are new and modified, truncated or chimeric to change their activities in comparison to wild Bt. For instance, there is 44% difference between the toxin in Bt176 and the wild corresponding Bt. All the modified Bt toxins have not been authorized seperately in food and feed, neither has been the wild Bt, and they have not been tested by themselves on animal and human health. Even if some studies have been performed, the receptors have not been cloned and the signalling pathways have not been identified, neither required for authorisations, the metabolisms of these proteins in mammals are unknown. Thus the argument of history of safe use of the wild Bt protein (not designed for direct consumption in contrast to several GMOs) cannot, on a sound scientific basis, be used for direct authorization of the above cited GM maizes, without in vivo chronic toxicity tests (Toxotest approach), as it is requested for a new pesticide. Some improvements may even be included with regard to pesticide legislation, since these toxins are continuously produced by the plant devoted for consumption.

The proteins usually compared (modified Bt toxins and wild ones) are not identical, and the tests on human cells of the new modified Bt proteins are not available. Nor are they requested by authorities. Their stability has only been assessed in vitro and maize proteins are never all fully digested in vivo. If some consumers suffer stomach problems or ulcers, the new toxins will possibly act differently; the digestion in children could be affected, and these GMOs could be eaten anywhere in the world.

2. *Differences between acute, subchronic and chronic tests and their potential. The Toxotest approach.*

The acute toxicity approach (less than a month of investigations on rodents) may be proportional to the dose and correspond to a rapid poisoning of animals, generally with force-fed experiments, with toxic effects on tissues. Many pesticide studies in the scientific literature detail some long-term side effects of pesticides at low doses, not apparent in short-term experiments. These are toxic for one or several levels in the ecosystem and their action may be only moderately specific. They are selected because of their capacity to disrupt the "cell web", i.e. to interfere with a signalling pathway, and this could be unspecific of the targetted parasite. For instance Roundup is known to disrupt the EPSP-synthase in plants, but is also known to interact with the mammalian ubiquist reductase (13) common and essential to cytochromes P450, a wide class of detoxification enzymes.

When there is a low-dose impregnation of the feed (with a pesticide-GM plant for instance), the chronic effects are more differential according to the sex, the physiological status, the age, or the rythm or length of intake in case of a drug. These are more determining on some pathologies development than the quantity of toxin ingested in one time. This is in part because they involve the liver, kidney and other cytochrome P450-rich organs, for long-term metabolism and detoxification, and this is hormono-dependent. It is also due to the process of carcinogenesis or hormono-sensitive programming of cells. The liver for instance is a sexually-differentiated organ for its enzymatic equipment. Thus any affirmation that would exclude an effect in subchronic or chronic tests, on the rationale that it is not dose-response related nor comparable in genders; this would not be scientifically acceptable. We are in the centre of the mechanism by which pollution, for instance, interferes with environmental health and risks.

One of the pivotal requirements is to understand the concept of dose-response and causality, which cannot be established with only two points. This is the case in the feeding trials described above and performed by the manufacturers with only 11 and 33% GMOs in the diets. This is true overall if no data has been obtained to test if the effect could be proportional to the dose. As we have emphasised, most of pathological and endocrine effects in environmental health are not directly proportional to the dose, with a differential threshold of sensitivity in both sexes. This is for instance the case in carcinogenesis and endocrine disruption.

It is impossible to conclude within only 13 weeks the kind of pathology that could be provoked by pesticide-GMOs and whether it is important or not. It is then necessary to prolong the tests, as suggested by EFSA (p. 50, Draft report on animal feeding trials) since at least one third of chronic effects visible with chemicals are usually new in comparison to subchronic studies. The so-called Toxotests, which are proposed to be at least in part chronic

ones, should be performed on three mammalian species, with at least one which is not a rodent, similar to those used for pesticides and drugs. However, the chronic feeding tests for GMOs cannot be based on the NOAEL and LOAEL approach. There are several reasons for that. There is not only one chemical but several unknown metabolites and components, in RR varieties for instance, enhancing toxicity by the fact that they are mixed together. There is also no possibility to increase the doses of GMOs in an equilibrated diet over an acceptable level.

The Toxotest is proposed, basically composed of an extension of the existing 90 day tests, but with several more doses (0, 5, 10, 15, 20, 25, 30 % GMOs for instance – today the equilibrated diets tested contain 0, 11, 33% GMOs). This would be in order to characterize scientifically the dose-response approach. The latter cannot be taken seriously with only two doses. Other details for the Toxotest will be described elsewhere. The end product is the best health protection possible of the population without real clinical trials possible, in our case, for ethical reasons. In addition to being the best toxicological approach, the Toxotest will also favour the biotechnology economy and European Community, because it is more expensive to address a population problem than to work with laboratory animals; it is also more ethical to work on rats and other mammalian experiments, in order to get pertinent information, than to give pesticide-plants directly to humans on a long term basis.

The health effects, if any are revealed by proper studies, Safotests or Toxotests, like those suggested in Table 1, could only be due to 2 possibilies. Firstly, they may be due directly or indirectly to a pesticide residue and/or its metabolites. The direct effect means the pesticide effect on the consumer; and the indirect means a metabolism disruption that it has provoked within the plant first. This could not be visible by a compositional analysis, such as those performed to assess substantial equivalence. The latter is not a well-defined concept (how many cultures during how many years, during what climate and to measure what precise parameters?). Secondly, they may be due to the genetic transformation itself, its method provoking either insertional mutagenesis or a new metabolism by genetic interference. This is why to separate intended effects (genetic trait) to unintended effects (biotechnology) a spiking of the control diet with the toxin is insufficient. It could work in case of direct action of the toxin in mammals, but on the contrary one could not conclude between an insertional mutagenesis and a specific metabolic action in the plant due to the toxin. Anyway, this is more a research question on the mode of genesis of an effect on health; and propositions to study this will be made elsewhere. It is not necessary for a conclusion of the Safotest or the Toxotest, which would rather suggest if positive to exclude immediately the corresponding GMO from food and feed.

3. Critical review of subchronic 90 d tests protocols and statistics approved by authorities. The SSC method.

A serious experimental design is based on a proper choice of the groups and balanced sample sizes. In several authorized GMOs the sample sizes appear inadequate: 10 animals per group for the measurement of biochemical parameters as it is done for MON 863, MON 810 or NK 603 for instance is a too limited size to ensure that parametric statistic methods used by the company are reliable. Moreover an important discrepancy between GMO treated rats (80) and the total number of animals (400) renders more difficult the evidencing of pertinent effects, and confusion factors are brought in at the same time with 6 different reference diets in addition to the 2 normal control groups. This introduces new uncontrolled sources of variability about the effect of the diet and not the GMO per se. The impact of multiple diets

could have been observed with only one new group of the same size than GMO groups eating a mix of 6 different diets.

Several questions have been raised by companies and authorities as well as comments on statistically significant effects that would not be biologically meaningful. A subjective part is introduced at this level because it is necessary to take into account the context and the general and detailed knowledge of toxicology and endocrine disruption, as EFSA underlines. This might be highly expert-dependent. This is why, to avoid or prevent misunderstanding, we propose in addition a new statistical approach based on classic methods to analyze the 90 day tests, comprising control and reference diets, called the "SSC method". It will be described in details elsewhere.

Briefly, after the necessity to model and analyze the growth curves, multivariate data analysis and data mining of all parameters can be used to correlate, cluster and select meaningful variables. Thereafter necessarily, the detailed comparison between GM-treated and control groups, fed with the near isogenic line, will be followed by the study of specific diet effects, when there are non substantially equivalent feed for reference groups. For that, the controls will be first compared by multivariate inference to reference groups, and thereafter similarly GMO-treated groups with reference groups. The significant differences linked to the GMO and/or the composition of the diet will be classified by organ and function. The results appear then more clearly than with the simple statistics accepted today by authorities, and reveal in addition new information, as it can be demonstrated.

As recommended by EFSA, an appropriate and relevant statistical analysis is a crucial point to be met. It then should be conducted in the several following steps, allowing the use of several methods according to the questions raised:

- Obtaining and modelling of the growth curves and feed consumption, estimation by non linear regression, validation, statistical comparisons in order to test if the curves are significantly different. This necessitates the use of time series analysis, selection models, non parametric tests, Akaike Information Criteria and related methods. Water consumption should also be an important factor to follow and understand better kidney and urine data.
- Study of dose-response prediction using non linear regression should be the goal, but the only 2 doses generally used in these tests do not allow to evidence linearity. Moreover, in the cases where there are not dose-related trends or relationships with these 2 doses, the absence of linear dose-response curves cannot be a reason to neglect the effects. For instance U or J curves may be characteristic of endocrine effects; spiky irregular curves may be detected in carcinogenesis.
- Simultaneous analysis of all observed variables: multivariate data analysis, principal component analysis, correlations analysis, factorial analysis, clustering.
- Multivariate comparisons of the different variables: hypothesis testing, multiple ways ANOVA, MANOVA and others to determinate if the group differs for the different questions: specific GMO effect or diet effect per se. To evidence a detail, when comparing 2 means, SEM should be calculated to determine confidence intervals, however SD have been used by the company for MON 863 and NK 603 files.

Apart from empirical curves in some instances, ANOVA and univariate hypothesis testing only on the GMO effect, none of the other statistical approaches is currently used nor requested by authorities.

4. Human tests and post-market monitoring (PMM)

As a matter of record, human tests are few. Moreover, epidemiological studies are not possible in America, since there is no organized traceability of GMOs on this continent, where, by far, most of edible GMOs are cultivated. In consequence, a post-market monitoring (PMM) is proposed in the population. The Cartegena Biosafety Protocol identifying GMOs at the borders of a country has now been signed by over 150 countries including those of the EU. As underlined by EFSA (p. 64, Draft report), PMM may have value in detecting unexpected adverse effects. It thus could be considered as a routine need. It is a later step which may inform risk management (p. 95, Draft report). It can be relied upon as a technique for monitoring adverse events or other health outcomes related to the consumption of GM plant derived foods, provided the Toxotest approach together with the SSC method had already been applied.

The traceability of products from animals having eaten GMOs is also crucial, because they can develop chronic diseases not directly known today. These are suggested by the hepatorenal toxicity possibly induced in some instances by GMOs. Their labelling is necessary because some pesticide residues linked to GMOs could pass into the food chain, and also because nobody would want to eat disabled or physiologically modified animals after long-term GMOs ingestion.

Conclusion

Transcriptomics, proteomics and other related methods are not ready yet, moreover they may be inappropriate to study toxicity, and could not in any way replace in vivo studies. By contrast they could well explain pathological results or mechanisms of pesticide actions, present in the GM plants, if found.

The transparency of crude data from toxicological studies is crucial. These can be put on line on the EFSA website in order to have a full review of these by the wider scientific community, in order to protect better the consumer. Since fundamental research is regularly published, it should be the same for this kind of applied reasearch on long term health effects, as suggested by the CE/2001/18 regulation.
It is unacceptable to submit 500 million Europeans and several billions of consumers worldwide to these new pesticide-GM derived foods or feed without more controls than today, especially given the evidence of worrying problems (Table 1). That is why we propose to improve the protocol of the 90 day studies together with the Toxotest kit (1 and 2 years long) which should be rendered obligatory, with sexual hormones assessed. The new SSC method is offered in addition. This should not be optional if the plant is designed to contain a pesticide (more than 99% of cultivated commercialized GMOs, 15), whilst for others, depending on the inserted trait, a case-by-case basis approach in the method to study toxicity will be necessary.

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