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DES ALIMENTS

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OPINION

of the French Food Safety Agency following its internal request concerning the article published in the 'International Journal of Biological Sciences' entitled "A comparison of the effects of three GM corn varieties on mammalian health"

THE DIRECTOR GENERAL

1. REVIEW OF THE REQUEST

On 23 December 2009, the French Food Safety Agency (AFSSA) issued an internal request following publication of the article entitled "A comparison of the effects of three GM corn varieties on mammalian health" in the International Journal of Biological Sciences[1] in December 2009.
In this publication, the authors reviewed data from three 90-day rat feeding studies carried out with the three genetically modified (GM) maize varieties NK 603, MON 810 and MON 863[2] . They concluded that these data highlight signs of hepatorenal toxicity.
The conclusions of the publication were likely to cast doubt over the safety of the NK 603, MON 810 and MON 863 maize varieties.

2. BACKGROUND

The published study follows an earlier publication by the same team in which a statistical analysis of the data from a 90-day rat feeding study of genetically-modified MON 863 maize showed that rats exhibited signs of hepatorenal toxicity (Séralini *et al.*, 2007).
This publication was examined in 2007 by several risk assessment authorities involved in process for authorisation to place on the market genetically-modified plants. All concluded that the publication did not provide any scientific evidence likely to call into question the earlier Opinions of these authorities on the safety of MON 863 maize, which is considered to be as safe as non-GM maize varieties (CGB 2007, AFSSA 2007, EFSA 2007, Doull *et al.* 2007).

AFSSA's previous assessment of the GM maize examined in the publication

NK 603 In 2003, AFSSA received requests concerning two marketing authorisation applications for the genetically-modified maize variety NK 603, one relating to the importing and food use of grains and derived products under Regulation (EC) no. 258/97 and the other for importing and feed use under Directive 2001/18/EC. For both applications, AFSSA had issued a final Opinion in which it stated that the consumption of maize from the NK 603 line presented no nutritional risks for humans and animals (Opinion of 13 January 2004, Request no. 2003-SA-0401).

[1] Spiroux de Vendômois J., Roullier F., Cellier D. and Séralini GE. (2009) A comparison of the effects of three GM corn varieties on mammalian health, Int. J. Biol. Sci., 5, 709-726.
[2] These data are those which were examined in the context of marketing authorisation applications for the genetically-modified maize varieties NK 603, MON810 and MON863.

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MON 810 The maize event MON 810 and its derived products intended for food and feed have been notified under Articles 8 and 20 of Directive no.1829/2003 and were listed in the Community Register in April 2005. They have not been assessed at AFSSA in this context.
In April 2008, when the application for renewal of the authorisation of existing product produced from this maize was submitted, AFSSA assessed the application and concluded that the maize variety MON 810 exhibited the same level of safety as conventional maize varieties (Opinion of 30 April 2008, Request no. 2008-SA-0043).
Following the report by Professor Le Maho and publication of the EFSA opinion of 30 June 2009, AFSSA has reasserted its conclusions (Opinion of 29 January 2009, Request no. 2008-SA-0266 and Opinion of 30 October 2009, Request no. 2009-SA-0257).

MON 863 The maize transformation event MON 863 was assessed by AFSSA in 2003 for its importation, processing and use just as for any other maize under Directive no. 2001/18/EC (Opinion of 6 November 2003, Request no. 2003-SA-0324) and under Regulation (EC) 258/97 relating to novel foods and novel food ingredients (Opinion of 2 December 2003, Request no. 2003-SA-0325).
These Opinions concluded that there was no risk for humans and animals associated with consumption of this maize.

3. EXPERT ASSESSMENT METHOD

The collective expert assessment was conducted by the Scientific Panel (CES) on Biotechnology, which met on 21 January 2010.

4. DISCUSSION

Review of the data analysed in the publication:

Nature of the genetically-modified maize

NK 603 maize is glyphosate-tolerant due to the tandem introduction of two genes allowing the CP4 EPSPS and CP4 EPSPS L214P proteins to be expressed (an amino acid substituted at position 214). The genes come from a common soil bacterium *Agrobacterium sp.* strain CP4. The first gene is controlled by the actin promoter of rice and the second by the 35S promoter of the cauliflower mosaic virus.
Both proteins are glyphosate-tolerant 5-enolpyruvylshikimate-3-phosphate synthases, which catalyse a reaction in the shikimic acid pathway leading to the biosynthesis of aromatic amino acids. The enzyme, found naturally in maize, is inhibited by glyphosate: the aromatic amino acids are no longer synthesized and this leads to the death of the plant. The expression of tolerant forms of the enzyme (CP4 EPSPS and CP4 EPSPS L214P) enables aromatic amino acids to be produced even in the presence of glyphosate. The enzymes expressed by the transgene are homologous to the enzymes naturally found in the plant. No toxic effect was demonstrated by administration above 572 mg/kg b.w. for CP4 EPSPS and 817 mg/kg b.w. for CP4 EPSPS L214P (acute toxicity for a single dose in mice).
The possible presence in maize of glyphosate and its secondary metabolites is due to treatment of plants with herbicide. The gene products provided by genetic modification do not change the structure of the glyphosate and its metabolites and no new metabolite is produced by the enzymes expressed by the transgene. Furthermore, the use of glyphosate on the NK 603 maize variety received two favourable Opinions from AFSSA (application references 2008-0630 and 2007-3111). When issuing these Opinions, AFSSA assessed in particular the toxicity of the glyphosate and its secondary metabolites. AFSSA confirmed that the glyphosate concentration in the treated maize is lower than the maximum residue levels set by the European Union.

The MON 810 and MON 863 maize varieties express respectively the toxic domain of the CRY1Ab and CRY3Bb1 proteins. The proteins are encoded by genes from *Bacillus thuringiensis* and have the ability to create pores in the intestinal cells of certain lepidopterans leading to the disruption of intestinal absorption. The CRY1Ab domain is toxic to the European corn borer (*Ostrinia nubilalis*) and the Mediterranean corn borer (*Sesamia nonagrioides*), whereas CRY3Bb1 is toxic to the larvae of beetles, especially the Colorado potato beetle (*Leptinotarsa decemlineata*) and beetle pests of maize roots (*Diabrotica sp*).
No signs of toxicity to mammals have been reported in the literature from among the many studies on these proteins (for review: Betz *et al,.* 2000, Bondzhio *et al.,* 2008).

Toxicological tests
Studies were conducted by Monsanto for NK603 and MON810 in June 2000, and by Covance for MON863 in March 2001.
In both cases, the studies met the requirements of Good Laboratory Practice (GLP), which demonstrates the application of internationally-accepted methodological principles and data traceability.
Two concentrations of GM maize (11% and 33%) in the diet were assessed in Sprague-Dawley rats. The control groups received near-isogenic maize at the same concentration. Six other groups of rats received maize from conventional non-GM varieties, at the highest concentration of 33%.
The NK603 maize used in the study was treated with glyphosate. The levels of glyphosate and AMPA (its main secondary metabolite) in the grains were respectively 0.09 µg/g and below the detection limit of 0.05 µg/g (NK 603 application, Monsanto).
Eighty-three parameters (biochemistry + weight) were measured and around 450 comparisons made for each of the three 90-day rat feeding studies.

ANALYSIS OF THE PUBLICATION

This analysis focused on two aspects of the publication: 1) criticism by the authors of the methodology used by Monsanto and 2) statistical analysis of the data and its interpretation by the authors.

1) Criticism by the publication's authors of the methodology

1.1 Number of doses and number of measurements

According to the authors, the OECD protocol was not followed for either the number of doses applied (three recommended) or the sampling times. The first remark is justified, although it should take into account the specific methodological issues related to the administration of the GMO. Indeed, the maximum dose was imposed in order to respect a balanced diet (33% in the case of this maize). The lower dose (11% in this case) enabled the possible relationship between the high dose and a lower dose to be assessed. The relevance of an even lower third dose appears highly questionable *a priori*.

Regarding the number of measurements taken over time, the applicant correctly followed the OECD protocol which recommends a haematology and clinical biochemistry examination "at the end of the test period" and "when any interim blood samples may have been collected" (OECD, 1998).

The authors noted that for groups receiving a diet containing a commercial reference maize variety, its substantial equivalence to the GMO maize was not demonstrated. In their opinion, studying several varieties of non-GM reference maize lines introduced unnecessary variability which creates an unbalanced experimental design as regards data from groups receiving GM maize and those receiving non-GM varieties. Although the number of groups should be taken into account in terms of statistical analysis, the CES experts saw on the contrary a major advantage to the use of commercial varieties, for measuring the variability of parameters with different non-GM plant-based diets.

1.2 Observed biological parameters

According to the authors, *"some important measurements ... were not conducted".* They cite:

- γ glutamyltransferase (γGT) (not measured at both sampling times),
- blood cholesterol and triglyceride levels,
- cytochrome P450 family members and pituitary hormones.

Concerning the cholesterol and triglycerides not measured in the MON810 and NK603 studies, the claim is justified.

The γGTs, such as the alkaline phosphatases (ALP) which were measured, are non-specific hepatotoxicity markers (Zimmerman, 1984; Bearman, 2000). As such, they are not the most relevant parameters.

It is important to respect the hierarchy of markers deduced from the experience. Thus it is accepted by EMEA (EMEA, 2006) that the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) enzymes, associated with bilirubin, are actually considered to be the most relevant signal of liver toxicity. The presence of macroscopic and/or microscopic alterations to the liver also reinforces the assessment (Guillemain, 2009). The OECD protocol recommends measuring more than two enzymes indicative of liver function, which was the case in the studies performed.

Concerning markers of endocrine function, their measurement may be of interest. They are however not required by the OECD 408 protocol (OECD, 1998). Finally cytochrome P450 family members are mainly measured in specific cases of interaction with the metabolism.

AFSSA, aware of the specific issues and methodologies related to the administration of the whole food, has initiated discussions aimed at optimising the OECD 408 protocol for the assessment of GMOs and examining in greater depth the aspects related to the statistical analysis of data.

1.3 Statistical power related to the experimental design

Each group consisted of 20 males and 20 females, with blood and urine tests concerning only 10 animals of each sex per group. Organ weights and histology involved the whole sample, i.e. 20 animals. The authors considered that "*the sample size [...] is largely insufficient to ensure an acceptable degree of power to the statistical analysis performed",* in particular for the biochemical parameters, which were only measured on a sample of 10 animals per group and per sex.

The statistical power of the test is an important concept, as it depends on both the sample and the effect size observed between treatments (set here by the authors at one standard deviation). The toxicological significance of a difference equal to one standard deviation depends on the parameter considered.

This calculation is also based on the variation of a single parameter, whereas the toxicological analysis takes into account a set of convergent parameters with toxicological significance for a tissue or an organ.

Having re-examined the data from the study of the MON 810 maize, we calculated that the power of a comparison test (ANOVA) based on a sample of 10 rats was 80% when the difference between the GMO treatment and the non-GMO treatment was equal to 1.3 standard deviation of the observations.

Using the same data, we obtained a power of 88% for an effect size of one standard deviation for the organ weight parameters which relate to a sample of 20 animals per sex and per group. This point will soon be dealt with in an AFSSA Opinion.

1.4 Interpretation of differences in effect between doses

The authors criticised the fact that the lack of a linear dose-effect relationship was used to assert the safety of the GMO. It is indeed meaningless to speak of a linear dose-effect relationship when the protocol only involves two doses. However, comparing effects at low and high doses is a relevant factor in the assessment. Thus, an increase in the effects observed between the low dose and the high dose strengthens the effect's biological significance. Conversely, an effect at the low dose which is considered to be significant in statistical terms but not in biological terms, and which is not confirmed at the high dose during a 90-day treatment period, would have little toxicological significance.

The authors also considered that:

- the duration of the study only enabled moderate effects to be revealed,
- the disappearance of the effect at the high dose or after a longer exposure time should not be used to rule out an effect in the treated groups.

However, it was shown that the vast majority of toxic effects appeared during the 90-day feeding study, with the six-month studies merely confirming the previous data (Betton *et al.,* 1994 AFSSA, 2008b). The authors' assessment required the nature and dose of the product administered to be taken into account.
Thus, nephrotoxic or hepatotoxic agents often exhibit their effect in the short term, as has been the case with certain medicinal herbs such as Wall germander (Laliberté and Villeneuve, 1996) or green tea that has led to a need for liver transplants for some people (Gloro *et al.*, 2005, Bonkovsky, 2006, Molinari *et al.,* 2006).

Note that the 33% dose administered to rats is equivalent to the consumption in humans of 2 to 2.5 kg of the aforementioned maize per day[3].

The second argument fundamentally cast doubt over the toxicological reasoning and the interpretation of the experimental toxicological results. This position might be accepted if it were justified by scientific arguments, related for example, to the presumed mechanism of action.

1.5 Interpretation of the inter-sex effect differences

The authors suggested that the inter-sex differences were frequently used to reject significant effects. This assertion is unjustified, the usual reasoning leading to a statistically significant event being rejected while taking into account the effect on the other sex is as follows: if an event is considered to be biologically significant for one sex, it is obviously taken into account. If an event is found to be statistically but not biologically significant for one sex, the fact that it is not found in the other sex (either in statistical or biological terms) means that the lack of biological and toxicological meaning of this isolated phenomenon can be asserted.
Variations in physiological or metabolic parameters are indeed sometimes related to sex, and differences between sex-dependent groups may be accepted if they are substantiated.

2) Analysis of the data analysis and its interpretation by the authors

2.1 New statistical analysis of the data by the authors

Three types of analysis are presented in the publication:

i. Analysis of the differences between
   - rats fed GM maize and rats fed isogenic non-GM maize (control feed)
   - rats fed GM maize and rats fed six non-GM reference maize varieties (commercial reference varieties)
   - rats fed isogenic maize (control feed) and rats fed six non-GM reference maize varieties (Three GM maize varieties and two doses (11% or 33%) were considered, i.e. six GM treatments. The differences between the GMO and the control feed were tested at both doses for each maize with around 80 parameters and in two stages (at 5 and 14 weeks).

ii. Results of a principal component analysis to study the effect of gender on groups of measured parameters

iii. A graphical analysis of the change occurring between "at 5 weeks" and "at 14 weeks" in several parameters (creatinine, triglyceride, chloride) in groups of rats fed GM maize or the control feed.

[3] Calculation: 20g of food consumed for a 200g rat or 100 g/kg, or 33 g of GMO maize/kg, which leads to 33 x 70 kg = 2.3 kg.

The authors performed many statistical difference tests (see Table E of the publication). Given that, it often happens that some stand out as significant. This is a common problem when many hypotheses are tested with the same data sample. As the authors point out, the statistical tests can be adjusted using appropriate methods (Hochberg and Benjamini, 2000; Benjamini and Yekutieli, 2001).
The authors corrected these p-values and noted most of the adjusted test results were no longer significant for all three studies. For the study concerning the MON 810 maize, the authors acknowledged that none of the results remained significant (page 711 last sentence). Tables 1 and 2 of the publication present the results without the p-values being adjusted for the false positive rate. The authors have used these tables in part to assert the presence of hepatotoxicity and nephrotoxicity. The only significant differences confirmed after p-value adjustment related to the group of male rats fed with a 33% dose of NK603 maize, on the urine phosphorus, lymphocyte and neutrophil parameters, out of the 83 parameters measured and the 452 comparisons performed.

The authors acknowledge that one significant difference is not enough to assess the effect of a diet including GM maize and that it is necessary both to assess the extent of this difference and to examine whether significant differences were also obtained between the isogenic (control) and the reference varieties. The authors address this aspect of the problem in two ways:

- by only reporting cases where any difference between the GMO and the control feed was greater than 5%,
- by boxing the value of the difference when this difference is significant only in the comparison between the GMO and the control feed, and not in the comparison between the control and the reference feed varieties.

Table 1 of the publication indicates that significant differences were obtained between the control and the reference feed varieties in the parameters mentioned above for NK603, i.e. urine phosphorus, lymphocytes and neutrophils, however these difference values were not boxed in the table. These results show that the effects observed and highlighted by the authors were not exclusively related to the GM maize-based feed.

Principal component analysis and kinetic plots
Principal component analysis (PCA) was used to demonstrate a gender effect (Figures 1-2). The effect of the treatment duration (5 and 14 weeks) was mainly analysed using plots (Figures 3-7). It would have been more rigorous to use a mixed model including fixed and random effects: the fixed effects here would be the duration of treatment, the sex factor and the diet, whereas the random effects would be used to model the variability between individuals.
The PCA was conducted to support the hypothesis that the response might differ in males and females. The plots shown first appeared to confirm this hypothesis, but the associated inertia is only 40 to 60%. The two axes of the PCA represented on the plot therefore leave much of the variability in the data unexplained.
Moreover, in the absence of a definition for the axes of the PCA, called factors 1 and 2 in the study, it is not possible to interpret the results of the PCA and, in particular, to identify the source of the differences between males and females.
Furthermore, the fact that there are differences between the genders is well known for certain parameters, but this does not necessarily imply toxicity (e.g. body weight, organ weight, some haematological and biochemical parameters).

2.2 Analysis of toxicological results

According to the authors, the results indicate hepatotoxic and nephrotoxic effects. AFSSA reiterates that these results (which were listed in the tables of the publication) do not take into account the p-value adjustment methods recommended by the authors.
Moreover, the values observed, particularly those for toxicity markers, should have been related to the data obtained from the commercial reference varieties and the historical data (see Table 2 of the publication by Doull *et al.,* 2007).

2.2.1 Results of the NK 603 study

Hepatic function

A significant increase in ALP was reported at the end of the study in females, at the 11% dose. This effect was not observed at the 33% dose and had no correlation with liver weight.

Renal function

The authors suggested a possible disturbance of renal function due to increased levels of P, Na and K in urine, in males at the 33% dose (Table 1) whereas the blood parameters for renal filtration did not confirm this hypothesis. The concentrations of minerals in urine are variable and depend in particular on water consumption. Alone, they are not sufficient to indicate kidney damage.

Urea and creatinine levels decreased at both concentrations, 11% and 33% (Table 1), whereas the reverse is to be expected in cases of nephrotoxicity.

Haematology

The 23% decrease in lymphocytes in females at the high dose is not linked to any other haematological parameter and has no macroscopic or microscopic effect on the target organs (e.g. spleen). The sporadic variations, which are not interrelated, do not allow any conclusions to be drawn regarding toxicology.

Organ weight

A small increase in heart and liver weight was observed in males at the 33% dose. Note however that the mean heart weight in this group (1.98 ± 0.261g) is identical to that of the GM 11% group (1.99 ± 0.254g) which itself does not differ statistically from the isogenic control group, nor does it differ statistically from that for the two groups receiving a commercial variety (1.93 and 1.92g for Cropland and Campbell, respectively).

These heterogeneous data, without the aforementioned major hepatotoxicity parameters being modified, are a perfect example of the lack of correspondence between the statistically significant variations and their biological significance.

2.2.2 Results of the MON 810 study

Hepatic function

The main observation was a slight decrease in serum albumin production in males at the 33% dose, resulting in a *de facto* reduction in the albumin/globulin ratio. This isolated observation has absolutely no toxicological significance. Nor was any histological alteration reported in this group.

Renal function

Here too, the conclusion of possible nephrotoxicity was only based on consideration of isolated parameters (increase in kidney weight and urea but in two different groups).

It should be noted that in Séralini *et al*. (2007), the authors used the significant reduction in kidney weight to suggest nephrotoxicity. This effect had not been observed in studies with the hybrids MON 863 x NK 603 and MON 863 x MON 810 x NK 603. This demonstrates that although the toxicological analysis should consider sporadic effects, the notion of convergence of arguments is essential.

Haematology

The number of leukocytes and lymphocytes decreased slightly in the female group fed MON 810 maize at the 33% dose. As the lymphocyte population is largely predominant in rats, it is logical that leukocyte levels were also modified. This is an example of cases where two parameters are reported as changed whereas in fact one of the changes is a consequence of the other.

Organ weight

The only significant variations in organ weight concerned the lowest dose for a single sex, which was not correlated with any biological effect.

2.2.3 *Results of the MON 863 study*

The data on MON 863 maize were already covered in a publication by the same authors in 2007. Following this publication, several authorities in charge of GMO risk assessments (CGB 2007, AFSSA 2007, EFSA 2007, Doull *et al*. 2007) decided to investigate the issue and reanalysed the data. All confirmed the absence of scientific evidence that would call into question the conclusions of the previous assessments of the MON 863 maize.

5. CONCLUSION

The authors based their conclusions on the results of statistical tests, whose shortcomings they criticised. They recommended using other methods that correct the risk of false positives. These methods lead to a lower number of significant differences.

The authors took into account heterogeneous results: biological constants and organ weights unrelated to the modified constants, different observation times, different dose levels, redundancy of certain parameters (absolute weight/relative weight of organs, ratio) without interpreting the toxicological relevance. The variations reported were sometimes in the opposite direction to what is usually accepted in toxicology as conclusive evidence of nephrotoxicity or hepatotoxicity.

Moreover, no reference is made to the histopathological examinations, which are however essential for corroborating toxicity in organs and tissue, particularly when discussing nephrotoxic or hepatotoxic effects.

Typically, analysis of data from a toxicological study is based on 1) the intrinsic significance of the variation in a parameter, 2) the meaning of this variation in relation to the alteration of an organ or system, and 3) the notion of convergence of events expressing an effect on a target. After taking these different factors into account in the studies of NK 603, MON 810 and MON 863 maize, AFSSA has not come to the same conclusions as the authors of the publication.

Consequently, the French Food Safety Agency considers that this publication does not provide any new evidence that would change AFSSA's earlier conclusions with respect to the safety of NK 603, MON 810 and MON 863 maize.

The Director General

Marc MORTUREUX

KEYWORDS

Keywords: GMO, NK603 maize, MON810 maize, MON863 maize, glyphosate tolerance, resistance to lepidopterans.

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