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DES ALIMENTS

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OPINION
of the French Food Safety Agency (Afssa)
on the study by Velimirov *et al.* entitled "Biological effects of transgenic maize NK603xMON810 fed in long-term reproduction studies in mice"

THE DIRECTOR GENERAL

The French Food Safety Agency (Afssa) carried out a self-tasking mandate on 21 November 2008, further to the study conducted by Drs Velimirov and Binter and Professor Zentek from the University of Veterinary Medicine Vienna (VUW) at the request of the Austrian Ministry of Health.
The maize NK603xMON810 on which the study was conducted was assessed by Afssa in 2004 and 2005 (opinion of 14/05/04 and 13/09/05).
Moreover, Afssa received a request from the Directorate General for Competition, Consumption and Fraud Prevention on 24 November 2008 to indicate whether this study was likely to cast doubt over the conclusions of the opinion of 13 September 2005.

Expert assessment method of the self-tasking exercise
The "Biotechnology" Scientific Panel examined this self-tasking mandate at its meetings of 18 December 2008 and 15 January 2009, when it proceed to the interview of two external experts.

Presentation of the study
This research aims to reveal any long-term effects on reproduction, under maximum exposure:

1- multigeneration study (MGS)
2- life-term study (LTS)
3- reproductive assessment by continuous breeding (RACB)

In these studies, three groups of mice were fed with diets that each contained 33% maize flour from either :

- transgenic maize NK603xMON810
- or a control maize, called isogenic, of the same genetic background not containing the transgenes
- or a variety of GM free Austrian maize.

The only significant differences between the groups, identified by the authors, are observed on reproduction in the RACB trial.

After consulting the "Biotechnology" Scientific Panel, the French Food Safety Agency issues the following opinion:

General comments on the study and implementation of the experiment
Before presenting the conclusions of the analysis, Afssa would like to make the following comments:

1- This is a study whose results have not been submitted for examination by the reading committee of a peer review.

2- It does not mention a follow-up of Good Laboratory Practices for the experiments carried out.

3- The protocol (RACB) implemented by this team does not comply with the official protocol defined by the National Toxicology Program[1]. As a result, the analyses on sexual behaviour, assays of reproduction hormones or histological analyses of the ovaries and testicles are not included in the study.

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[1] Chaplin RE and Sloane RA, 1996, Reproductive assessment by continuous breeding: Evolving study design and summaries of eighty-eight study. Environmental Health and Perspectives, 102, supplement 2 : 1-7.

4- The historical data of the mouse strain used in the experiment has not been provided as recommended by the "*Guideline on risk assessment of medicinal products on human reproduction and lactation, from data to labelling - EMEA 2005*".

5- The choice of an outbred mouse strain (OF1 strain) does not appear sufficient to demonstrate an effect on reproduction. The outbred mice populations are not genetically defined at individual level and, in such conditions, the genetic structure of a limited size batch (as is the case here) can vary considerably.
Moreover, in analysis concerning several generations, the inbreeding coefficient can increase randomly during the experiment and not uniformly between the three test groups. The "fertility" character can obviously be affected.
In this case, the authors should have used either inbred mice[2] or significantly increased the number of animals per group.

6- The origin of the maize used as an isogenic comparator is not clearly defined, and these types of maize are no longer on the market.

7- The choice of a single proportion of 33% of maize grain in the diet is not sufficient, when it is recommended to use two doses in the 90-day toxicity test in rats[3] in order to demonstrate a dose-effect.

8- The study does not provide raw data of the different experiments. It is therefore impossible for an external expert to redo a complete statistical analysis of the test results. Access to these data was requested, through the Austrian Agency for Health and Food Safety, from the Austrian Ministry of Health which commissioned the study. It has not been possible to meet this request.
In addition, the animal testing conditions as defined in the "Standard Operation Protocol" have not been described in the study.

9- Several calculation errors have been identified in Tables 36 (page 39) and 59 (page 77). The comments on the consequences of these errors are reported in the paragraphs below.

Comments on the results

Analysis of the chemical composition of maize grains and diets
The analysis of the chemical composition of the different types of maize used in the experiment reveals significant differences in the concentration of zinc, copper and vitamins A and E. However, these compounds are described in the literature as being involved in the fertility of animals[4].
The chemical compound analysis is inadequate and does not follow the OECD's recommendations of 2002, specific to maize[5].
Data on the anti-nutritional and toxic factors of maize (phytic acid, para-couramic acid, furfural and trypsin inhibitors) as well as a statistical data analysis, would have been necessary to conclude on the substantial equivalence of the different grains used in the experiments.
We also note that the samples of ISO grain are not pure. The 35S promoter DNA has been detected by PCR in the batches of ISO grains. This promoter is specific to the transgene, and therefore to GM grains (table 14, page 28). Lastly, the levels of mycotoxins and microorganisms vary from one batch to the next.
These discrepancies in the samples' preparation may have effects on the reproduction of the mice. No comment on the matter were done by the authors.

Multigeneration study (MGS)
The study focuses on four successive generations (F1 to F4) from the parents F0. In the first analysis, the animals fed on GM maize (GM) were compared to those that were fed on isogenic

[2] Chia R, Achilli F, Festing MF W and Fisher EMC. 2005 The origins and uses of mouse outbred stocks. Nature Genetics, 37, 1181 – 1186.
[3] EFSA GMO Panel Working Group on Animal Feeding Trials, 2008, Safety and nutritional assessment of GM plants and derived food and feed: the role of animal feeding trials, Food Chem Toxicol. 46, 1, S2-70.
[4] Teliiman S, Cvitkovic P, Jurasovic J, Pizent A, Gavella M and Roit B, 2000, Semen quality and reproductive endocrine function in relation to biomarkers of lead, cadmium, zinc, and copper in men, Environmental Health Perspectives,108,1.
[5] OECD, 2002, Consensus document on composition and consideration for new varieties of maize (*Zea mays*). Keyfood nutrients and secondary metabolites.

maize. The second analysis of the same parameters involved comparing the animals fed on isogenic maize (ISO) to those fed on conventional maize (Austrian cultivar).

The authors made statistical comparisons on sub-groups defined by size of litter (above or below 8 pups). No information has been provided to justify this limit of 8.
The results presented in Table 36 are fundamental in this study as the arguments and conclusions are based upon them. They may be questioned from several points of view:

- this table contains calculation errors (sixth column 1.20 instead of 2.95),
- the authors have calculated the average number of pups at birth, reported for all pairs, without taking account of the number of births per group (some pairs had no offspring). The number of pups should have been used as the denominator rather than the number of pairs tested.
- it shows an at times high death rate (18 to 24%) between birth and weaning in all groups. This is abnormal for OF1 mice and should have been explained.
- the number of pups (born and/or weaned) per litter reduces as the generations progress. The low reproduction rate of the ISO and GM groups in the fourth generation, 27 and 36% respectively, reduces the sample which was already small at the start of the experiment.
- the data varies considerably, which is to be expected with outbred lines.

The histological analyses do not reveal any differences between the organ structure of mice fed with GM maize and that of the control mice. These results confirm the absence of effect of food containing GM maize on the structure of these organs.
Transcriptomic analysis is complete and well conducted. The functional analysis of the differentially expressed genes is realized in order to identify the modified metabolic ways. However, it is difficult to relate a difference in the transcriptome of the intestinal cells to a particular diet and to conclude on an effect from the presence of GM-maize in the diet. Moreover, these results are not connected to reproductive function.

Life-term study (LTS)
Ten females per group from the F1 generation born from dams already fed on GM maize or on isogenic maize, received the same diet as their parents throughout their lifetime. No statistically significant difference is observed on the survival curve due to the reduced animal numbers and the occurrence of pathological events (cancer) associated with the genetic strain used (OF1), and therefore unrelated to the treatment.

Reproductive assessment by continuous breeding (RACB)
Twenty four mice breeding pairs from F0 were formed and allowed to produce four successive litters. The animals fed with the food containing GM maize (33%) were compared to those that were fed with isogenic maize.
Unlike the initial RACB protocol, where the pups were sacrificed at birth, they were kept with their parents for 3 weeks until weaning, except the last litter. However, with this type of outbred line, the males become aggressive with age and the females are likely to eat their own pups. These known facts are not documented.

According to the authors, this study is the only one to reveal an effect of a GM maize-based diet on the 3rd and 4th mice litters (Table 59, page 77). Table 59 does, however, contain a calculation error on the line “number of pups at birth/pair” in the GM group of the 4th litter. This number has not been divided by the number of successful pups but by the number of mice pairs tested. This error led to a false statistical analysis and interpretation, which therefore casts doubt over the main conclusions of the study. Table 1 below presents the corrected data.

Number of pups at birth/pair	1st litter		2nd litter		3rd litter		4th litter	
	ISO	GM	ISO	GM	ISO	GM	ISO	GM
Study data	9.00	8.22	10.83	10.65	**11.92***	**9.68***	**11.38***	**8.21***
Corrected data	9.00	8.22	10.83	10.65	**11.92***	**9.68***	11.38	*9.85*

Table 1: correction of Table 59, page 77. *significantly different ($p<0.05$).

After correction, we note that the differences between treatments are smaller. The difference between the GM group and the ISO group is no longer significant for the 4th litter[6].

In addition, in this RACB experiment, we note a lower death rate among pups during suckling in the GM groups than in the ISO groups in general (except for the 2nd litter), which is contrary to the authors' conclusions.

We also note a significant number of pairs that do not produce pups from the start of the experiment. The cases of sterility observed in females have not been analysed in pathological terms. It was essential to carry out an autopsy in each case to determine sterility causes. This involves an in-depth analysis of the genital organ (ovary histology, follicle and oocyte count and so on) and analysis of the endocrine parameters (measurement of sexual steroids in the blood). Vaginal atresia is a very common anormality in mice and can be detected easily. It is evidently not compatible with mating and gestation. It is surprising that no explanation has been given for non-inbred mice pairs not reproducing.
As in the previous experiment, we note a high death rate between birth and weaning, especially for the 4th litter (14 and 12%). No explanation has been given.

The fluctuation observed in the number of mice born within a group shows the inter-group variability associated with the "outbred" character of the mice. This tends to suggest an environmental effect and highlights the interest in working with a large number of pairs.

Based on the results of the RACB study, the authors concluded that there was a link between the GM maize-based diet and reproductive traits of the mice. But after the corrections, some of the statistical differences that had been revealed are no longer significant. This casts doubt over the main conclusions which, moreover, should have been confirmed by additional studies such as:

1) a similar study in which the pairs were swapped to demonstrate if the reduction in fertility was linked to the male or to the female, as required by the RACB protocol;
2) a hormonal study like the assay of LH, FSH, GnRH, prolactin and leptin, as well as testosterone and oestrogen, to try to understand the causes of reduction in fertility;
3) a histological analysis of the ovaries and genital tract of the females;
4) a statistical per variance analysis of the results enabling "treatment x order of litter" interactions to be tested for.

Conclusion of the French Food Safety Agency (Afssa):
The French Food Safety Agency believes that the data presented in this study does not support the conclusion that a diet of NK603xMON810 maize affects reproduction in mice. Such data does not cast doubt over the food safety of this maize grain and its by-products, and therefore over the conclusions of the opinion of 13 September 2005.

Key words: NK603 maize, MON810 maize, mice, reproduction.

[6] The statistical processing of values for the 4th litter has been repeated by our experts, with account taken of the values of the standard error presented in the study.