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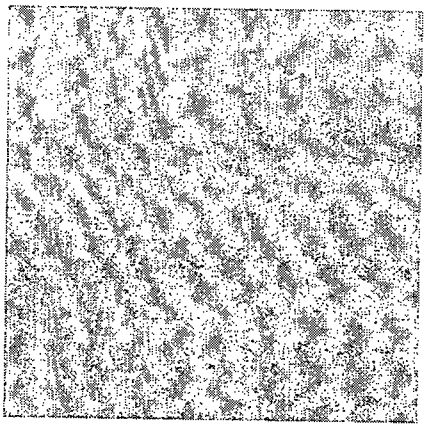
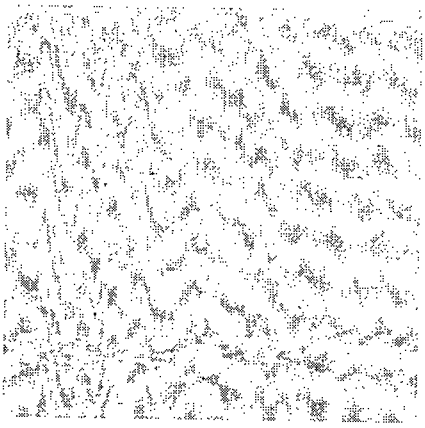
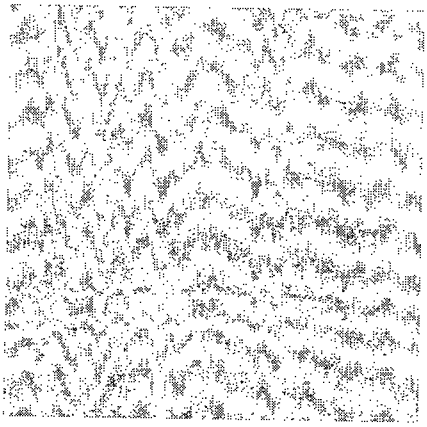


Report

Biological effects of transgenic maize NK603xMON810 fed in long term reproduction studies in mice





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Biological effects of transgenic maize NK603xMON810 fed in long term reproduction studies in mice

11. November 2008

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Contents

Abstract

The aim of the study was to examine effects of the stacked GM crop NK603 x MON810 in different models of long term feeding studies. So far no negative effects of GM corn varieties have been reported in peer-reviewed publications. But the hypothesis, that effects after long term exposure might become evident in multi-generation studies has rarely been investigated.
In this study three designs were used, including a multi-generation study (MGS), a reproductive assessment by continuous breeding (RACB) and a life-term feeding study (LTS), all performed with laboratory mice (strain OF1). The test diets differed only as to the inclusion of 33% NK603 x MON810 corn (GM) versus non-GM corn of a near isogenic line (ISO), both grown under identical conditions in Canada. The MGS also included one group with a non GM corn cultivated in Austria (A REF). All corn varieties used in the MGS and LTS were harvested in 2005, the transgenic and isogenic corn for the RACB were harvested in Canada in 2007. No Austrian corn was used in this case. In the MGS microscopic and ultrastructural investigations were performed to detect changes at the organ and cell level. Gene expression patterns were compared by micro array expression profiles of the intestine as feed-animal interface and by real time PCR.
The results of the MGS showed no statistically significant differences concerning parental body mass. The number of females without litters decreased with time in the GM and ISO group, especially in the 4th generation. In the group fed with A REF corn fewer females were without litters, and accordingly more pups were weaned. The production parameters average litter size and weight as well as number of weaned pups were in favour of the ISO group. These differences were also seen in the RACB design and were statistically significant in the 3rd and 4th litters. In addition, the inter-individual variability was higher in the GM group as compared to the other groups.
The LTS showed no statistically significant differences in the survival of 3 groups of mice fed the different maize varieties.
In the MGS the continuative investigations revealed differences between the GM and ISO groups. The comparison of organ weights did not indicate directed dietary effects, except for kidneys. The electron histological investigation of the cell nuclei revealed differences as to fibrillar centres, dense fibrillar components and the pore density in hepatocytes. This could point to an effect of the GM crop on metabolic parameters. Immunohistochemistry revealed no systematic differences in CD3, CD20 positive cells and macrophages in gut tissue. The microarrays showed differences between the feeding groups. When the data of both non-GM feeding groups from MGS were combined and compared to the GM feeding group, the discrimination became more evident. Analyses of metabolic pathways indicated, that the groups differed regarding some important pathways, including interleukin signalling pathway, cholesterol biosynthesis and protein metabolism.

Summarizing the findings of this study it can be concluded, that multi-generation studies, especially based on the RACB design are well suited to reveal differences between feeds. The RACB trial showed time related negative reproductive effects of the GM maize under the given experimental conditions. The RACB trial with its specific design with the repeated use of the parental generation is a demanding biological factor for the maternal organism. Compared to the findings in the RACB trials it can be assumed that the physiological stress was considerably lower in the MGS trial. The trial design of using "new" parental generations instead of continuous breeding with the same generation has to be considered as being obviously less demanding. This might have masked the impact of dietary

factors on reproductive performance. However, this part of the experiment is valuable as such because it underlines the need for different experimental designs for the assessment of dietary effects that have an unknown impact on animals. The outcome of this study suggests that future studies on the safety of GM feed and food should include reproduction studies. Physiological and genomic traits and depending on the nature of the genetic modification proteomic and metabolomic methods might be taken into consideration as additional tools to the tests performed in this study.

Parental reproduction

During the 20 week period of the RACB 4 litters were bred. From 24 pairs assigned to the ISO and GM group, all females of the ISO group (100%) delivered 4 litters (Table 59). In the GM group the number of deliveries declined with time. In the 4th litter only 20 deliveries occurred (p=0.055). The average number of pups born was always lower in the GM group but not significant before the 3rd delivery. There were significantly fewer pups born in the GM group in the 3rd (p= 0.011) and 4th (p=0.010) delivery and weaned in the 4th litter (p=0.025).
Regarding all deliveries per group more pups were born in the ISO than in the GM group (1035 versus 844). Furthermore females of the GM group always had smaller litters (n≤ 8) as compared to females of the ISO group (Figure 18).
At weaning the GM group had significantly fewer pups weaned in the 4th litter, though less pups were lost during weaning in all generations (only significantly in the 3rd litter p=0.025). Litters with a high number of pups tended to lose more pups. No difference was seen in the birth interval of 1st, 2nd and 3rd litters (data not shown).

Inter-litter comparison within the ISO group showed significantly less pups born in the 1st than in the other three litters and in the GM group significantly less pups were born in the 1st and 4th litters.

4. Discussion

Aim of the study

The aim of the study was to examine chronic feed effects of the stacked GM maize NK603 x MON810 in mice. A short term broiler study showing no effects had been conducted with the event in question, but no rodent feeding study was performed, since both parental GM lines had been declared safe and the new event was obtained by conventional breeding. No further transgene has been introduced.
Toxicological risks of GM plants are currently assessed by 90 day feeding studies with rodents. A 90 day study is considered as sufficient to detect adverse effects and the duration is considered as long enough by the EFSA GMO Panel. However, chronic effects might only become evident in longer lasting multi-generation studies, since reproduction and lactation as well as growth and survival rate of the offspring are very sensitive parameters. Furthermore almost all present GM crops are used for the nutrition of breeding animals.

Therefore the impact of dietary factors on fertility needs to be investigated in more detail. This is the first study investigating a stacked event in a multigeneration study focussing on mice in reproduction and development. Additionally microscopic investigations (histology, electron microscopy and immunohistochemistry) were performed to investigate possible effects of transgene maize at cellular level and microarray analyses for possible impacts at molecular level.

Methods

In this project two breeding designs were applied for the evaluation of the stacked event NK603 x MON 810 to highlight and compare the suitability of different study designs for risk assessment. The first experimental design was a multigeneration study (MGS) with 4 generations of mice. The second breeding scheme was a reproductive assessment by continuous breeding including 4 litters (RACB). Traits investigated were body mass development of parents and offspring as well as the fertility parameters litter size and survival rate until weaning.
To corroborate the results of the feeding studies additional investigations have been included in the MGS. Organ weights, histological and electron microscopic ultrastructural investigations were performed to detect changes at the organ and cellular level. Focus was laid on the intestine as a primary indicator of feed-animal interface. Immunohistochemistry was applied for the investigation of immune cells in the small intestine and finally gene expression profiles of the jejunum were performed by microarray analyses and q-RT-PCR.
Finally a life term study was performed with focus on mortality of mice allotted to the feeding groups. This design turned out to be less suitable for risk assessment studies.

For all trial designs, animals from an outbred mouse strain were chosen. The results obtained from an outbred strain can be considered as basis with a wider

range of various mouse genotypes. The alternative would have been to use inbred mice strains. Due to a lower genetic variability the results might have been less variable. However, the disadvantages of such an approach are also obvious. Inbred lines may be more or less susceptible to certain external stimuli and may therefore give a biased insight into the nutrition host interaction. This might happen in both directions, making the assessment of nutritional factors more complicated.

The results presented in the study provide a wide range of differences between the feeding groups that appear higher than natural variations normally expected. The total spectrum of methods is broad and should allow a valid conclusion about the potential impact of the different corn varieties on the animal. However, even with such a broad approach subtle effects might have been missed. On the other hand, some methods would need to be explored in more detail in future studies to evaluate the background and the variability under varying dietary conditions and with a broader spectrum of different mice strains or animal species, ideally covering several nutritional types (omnivorous, herbivorous, carnivorous).

Corn used for the feeding trials

In the MGS three feeding groups were established with diets containing the stacked corn NK603 x MON810 (GM group), the near isogenic line (ISO group) grown in Canada and an additional Austrian GM free reference corn (A REF). The addition of A REF corn was prompted by a slight contamination of the ISO corn and fulfilled the criteria of substantial equivalence. All different varieties were harvested in 2005. The RACB investigation included only the GM and ISO corn from a second harvest in Canada in 2007. All corn varieties were substantially equivalent in both harvests. The diets were offered as meal instead of pellets in order to avoid potential changes of feed components due to the application of heat and pressure. This is an important fact because for GMO crops the heat sensibility and in general the susceptibility to feed and food processing methods has hardly been addressed up to now.

Reproduction and performance- MGS

The MGS over 4 generations did not show significant differences between the feeding groups ISO and GM. The number of pups weaned, the average litter size and weight at weaning tended to be lower in the GM group as compared to the ISO group. At the same time the pup losses were higher in the GM group. These differences were consistent over the generations, but not significant, since the intra-group variability was very high.
It might be speculated that not all mice were compromised by the GM feed because of the high genetic variation between the test animals. The effects on litter size and weight became more notable in the 4th generation. In terms of production profit the ISO group had more weaned pups, 9% more females with litters (64% vs 73%) and slightly higher average litter weights at weaning in the ISO group (92.6 g vs 102.1 g).

The additional A REF group excelled in number of females with litters (91%) and accordingly more pups weaned as well as a 35% higher body mass production as compared to the GM group. Within four generations bred in the MGS no adverse effects on overall health and reproduction as well as performance were seen. Feed intake, fertility rate and number of pups born and weaned as well as body weight gain showed no statistically significant ($p<0.05$) differences.

Reproduction and performance- RACB

The 1st litters in the RACB displayed no differences between the GM and ISO feeding groups. Comparing the 2nd litters a very slight tendency towards smaller litter size and accordingly lower average litter weight in the GM group could be observed. In the 3rd and 4th litters the aforementioned traits became significant ($p<0.05$). Apart from a decline of deliveries, in the 3rd and 4th litters significantly fewer pups were born and in the 4th litter also significantly fewer pups were weaned in the GM group. The average litter weights were in favour of the ISO group with significant results in the 3rd litters at birth and weaning as well as in the 4th litters at birth. But in contrast to the MGS the loss of pups was higher in the ISO group. These results substantiate the assumption that long term feeding studies with more generations are useful in studying chronic diet related effects. According to our data the RACB design was better suited than the MGS, since the differences between the feeding groups were at significant levels. The biological phenomenon observed in the RACB trial cannot be explained by different nutrient intakes, because both diets were covering the energy and nutrient requirements and fulfilled the prerequisite of nutritional equivalence. Lower reproduction performance can be considered as indicator for a dietary effect. It can be speculated, that this effect was caused by a factor beyond nutrient supply. Whether this can be related to one of the two genetic modifications in the transgenic material or whether this is an unintended effect in the strict sense related to the stacked events has to be further evaluated.

Compared to the findings in the RACB trials it can be assumed that the physiological stress was considerably lower in the MGS trial. The trial design of using "new" parental generations instead of continuous breeding with the same generation has to be considered as being obviously less demanding. This might have masked the impact of dietary factors on reproductive performance. However, this part of the experiment is valuable as such because it underlines the need for different experimental designs for the assessment of dietary effects that have an unknown impact on animals.

The genomic work that was performed in the gut tissue of the mice of both groups is not indicative. However, the high number of deregulated genes that has been identified as difference between both groups could indicate a complex nutrition-host-interaction. This has to be further evaluated and gene expression profiles need to be considered in other organs and especially in the reproductive system. To date, trials have not been performed on that issue in feeding studies with genetically modified corn to our best knowledge.

Reproduction and performance in other trials

It is surprising that despite the long use of Bt corn since 1996 and the many controversial discussions about its safety, partly fuelled by anecdotal evidence, only few peer-reviewed multi-generation studies investigating potential effects of delta endotoxins on rodents have been conducted so far. Brake et al. (2004) used mouse testes as a sensitive indicator of potential toxic effects of diets containing Bt corn. The type of delta-endotoxin was not mentioned nor the conditions under which the diet was processed. This is an essential point when comparing different studies. When heat is applied during feed processing (e.g. pelleting), the danger of denaturing the transprotein is high and the outcome might be completely different compared to the raw material.
In the aforementioned Brake-study different mouse strains were used and crossed. For a short term study the mice were obtained at the age of 5 weeks and kept for 3 weeks on a conventional mouse chow. Only at the time of breeding the test diets were given. For the long term study with four generations 16 randomly chosen males and females (2 of each sex and strain for each diet) were used at the start and fed the test diets before mating. To produce the 2nd and 3rd generation 6 females and 3 males were paired for each strain and diet.
No diet related differences in the sperm development were found in this study. Significant differences occurring during the spermatogenesis were attributed to age differences. The progeny born within the same 24 hours was considered the same age. The authors also mentioned effects on litter sizes and weights. In the 4th generation they found significant differences in body weight comparing 3 animals / treatment at day 26 in favour of the GM diet ($p=0.001$) and on day 63 in favour of the conventional diet ($p=0.005$). It is also stated that litter sizes were similar in both feeding studies, suggesting that the Bt diet is not a factor impairing reproductive performance. The results are not corroborated by the present study. Data cannot be directly compared to the present results since inbred mouse strains have smaller litters and often have lower body weights.
A three generation study with Bt corn was also conducted with laboratory rats. Apart from some significant histopathological changes in liver and kidney no differences were found between the feeding groups (Kilic and Akay 2008). No differences concerning developmental performance were reported. But the number of offspring was generally very low in this 3 generation study, 4-5 pups / dam, whereas 10-12 pups / dam can be expected from Wistar Albino rats.
Many short term feed conversion studies with GM crops conducted with farm animals showed no negative effects (Aumaitre 2002; Flachowsky et al. 2005). The number of feeding studies with rodents is small, and inconsistent differences make it difficult to draw an overall conclusion on the tested GM feed (Hammond et al. 2006). Thus the safety of NK603 x MON810 is based on one poultry study performed by the applicant with the parental lines including 90 day rodent studies, and one poultry study with the stacked event (ACRE 2004). The GMO Panel of EFSA considers it unlikely that NK603 x MON810 maize will have any adverse effect on human and animal health (Opinion of GM Panel, 2005)

Regarding the weight development of the parental mice in the present study the short term feeding results can be corroborated, since the weight differences observed were very small and inconsistent. Chronic effects are difficult to measure and cannot be assessed by feeding trials in non performing animals. To ascertain that no chronic health impacts are caused by GM feed components the animal homeostatic system has to be challenged, since health is defined by the ability to

handle and overcome challenges, e.g. infections or stress, successfully. In the present study reproduction was chosen as a high performance status in a long-term feeding study encompassing several generations (MGS) and continuous reproduction of several litters (RACB). The RACB test design is normally applied for testing xenobiotic substances such as pesticides to define safety limits and has never been used before in connection with GM assessment to our knowledge. Since in toxicity tests, the LD50 for Cry1Ab showed no dose related deaths at an amount of 4000mg/kg (oral), the EPA has established the rule of an exemption from the requirement of a tolerance for residues of the plant pesticide active ingredients Bacillus thuringiensis Cry1Ab delta-endotoxin and the genetic material necessary for its production in all plants (EPA, 2001). But no multigenerational studies with the toxins have been performed to exclude any possible chronic effects.
The present RACB has been designed as whole feed study. The interpretation poses difficulties since it does not concern one single compound in different concentrations, but whole feed effects. On the other hand realistic conditions are reflected. Further studies are needed comparing GM corn producing the Bt toxin with non-GM corn spiked with corresponding amounts of Bt toxin to investigate whether the method of GM and/or the toxin are responsible for the outcome.

Organ weights and microscopic investigations (histology, immunohistochemistry, ultrastructural investigations)

Organ weights were recorded as potential indicator of a dietary effect on the organism. Liver and kidneys are central metabolic organs and are important for metabolic and excretory processes and are therefore often regarded as indicator organs for toxic effects. Therefore differences in liver and kidney weights are considered as sensitive risk parameters. Kilic and Akay (2008) also referred to significant differences in these organs.
Significantly lower relative kidney weights were found in GM females (F2, F3, F4) and in GM males (F3). Hammond et al. (2006) also mentioned lower relative kidney weights for MON863 (Cry3Bb1) fed males compared to the controls, but not at a statistically significant level. Microscopic pathological changes were described earlier in kidneys from rats from a 90day feeding trial, but they were not considered being feed related. A revision of these data indicated the possibility of GM-linked renal toxicity in male animals (Séralini et al. 2007), however, these results were critically discussed by several other authors including EFSA. Increased liver weights in females fed a GM diet were discussed as potential risk indicator (Séralini et al. 2007). In the present study liver weights were different between feeding groups in GM females, however, this was not unidirectional and therefore not interpretable. No differences in liver weight were seen in males.
The spleen is an important immunological organ and thus may also reflect dietary impacts. In the present study the relative spleen weights were significantly higher in the GM males of the F2 generation, in the other trial periods no such differences were found. No histological changes were seen in these organs.

The investigation of T- and B- lymphocytes as well as macrophages by immunohistochemistry did not reveal differences between the groups.

The ultrastructural investigation revealed some statistically significant differences between the groups. The fibrillar centres (FC) and dense fibrillar components (DFC) and the pore density are linked to the metabolic rate of cells. Increasing

metabolic rate leads to higher values of these parameters (Schwarzacher and Wachtler 1993; Dzidziguri et al. 1994). The nuclear shape irregularity, a way to detect enlarged surface areas, sometimes appears with rapid nucleus activity enhancement (Malatesta et al. 1998). Regarding the main test groups ISO and GM some differences were found. The lower nuclear pore density and the lower quantity of the nucleolar components FC and DFC in both females and males, found in hepatocytes of GM mice, indicate a lower liver metabolic rate in animals fed the GM feed. Similar findings were reported previously (Malatesta 2002). Since hepatocytes are involved in numerous metabolic activities, the cause of these observations is not clear.
The spleen lymphocytes in male mice showed higher DFC values in the GM group compared to the ISO group, suggesting an increased activity. Females seemed not to be affected. The DFC in pancreatic cells was decreased in males of the GM group, the FC was slightly increased.

Therefore, a generalizing conclusion about cell activities is not possible. The comparison of the ISO group to the REF A group showed only few differences. Only the decreased FC and DFC values in hepatocytes of male mice in the A REF group as compared to the ISO group were significant. The other findings showed comparable values.
Although the ISO and A REF diets were based on different corn varieties, the ultrastructural data of these two groups are closer together than those obtained from comparing the ISO and the GM group. Possibly, these parameters are less influenced by the maize variety than by the genetic modification.

Molecular analyses- Microarray and q-RT-PCR

Differences in gene expression in the intestinal tissue were seen in a number of biological processes when the different groups were compared. The corn might have contributed to that because the substantial equivalence was given, however, minor differences might have acted as extrinsic factors. The inter-individual differences generated by the outbred strain (intrinsic factors) may have amplified noise of the expression data. Microarray data display phenotypic variability through noise from intrinsic or extrinsic sources and can make those data difficult to interpret (Raser and O'Shea 2005). When ISO and A REF were pooled to one group and compared to the GM group, the expression data from ISO and GM comparison became more pronounced and the level of significance increased in the pathways protein biosynthesis as well as protein metabolism and modification. In addition to difficult data interpretation through noise the majority of differences found in the array data were under a fold change of 2 which is rather low and near detection limit of microarray analyses. Moreover, the dynamic range/sensitivity of microarrays limits their use in detecting changes in mRNA levels of those genes expressed at low abundance (Lord et al. 2006). Clustering into biological processes and pathways was used in our study to overcome this limitation.

Influence of the variety but also of the genetic modification were observed in microarray analyses of jejunal tissue. The intestine is considered as "feed-host interface" and until now no effects of Cry1Ab on mammalian intestinal cells were reported (Bondzio et al. 2008). As there are no previous pathways identified whole transcriptome microarray analyses covering the whole murine genomic

profile were used as a pre-screening tool. Significant ($p<0.001$) differences in gene expression were identified in a number of biological processes and pathways between the GM and non-genetically modified diets.

Q-RT PCR, the gold standard for gene expression analysis, was done with a set of 45 out of 400 deregulated genes previously identified by microarray analyses. From the 45 genes investigated on the TLDA, 9 ($p<0.05$) genes were classified as deregulated with influences by the trial, but also by sex and anatomical site. Differences between ISO and GM fed mice detected by microarray analysis were observed in several pathways. Clearly more work needs to be done on those analyses to get further insights into natural variation of gene expression and potential impact of dietary modifications. In a next step more work is necessary regarding the normalization of expression data and extending set of target genes that can contribute to the list of deregulated genes by GMO food. The networks around the marker genes identified are a promising issue of further research. Further the sampling strategy has to be improved to circumvent high intra-group variability.

Variability trough noise is coming from extrinsic e.g. the corn varieties and intrinsic, e.g. outbred strain sources that make array data difficult to interpret (Raser and O'Shea 2005). Further work is necessary to confirm and identify the full set of deregulated genes, identify involved pathways and especially to proof the deregulation at protein level. So far the genes detected represent only a proof of principle that differences can be seen between the ISO and GM group but no statement about the meaning of those genes can be discussed.
Due to the high technical demands and costs of such trials it will be difficult to set up these approaches in the future regarding the high number of new applications for the import of transgenic foods and feeds into the EU that are expected to come. However, based on the experience of this study it seems to be feasible to establish new test models that would increase consumer safety in this important area of risk assessment at least in selected GM feed and food materials.

5. Conclusion

Feeding mice with diets containing the transgenic corn NK603 x MON810 in different models of multi-generation studies indicated that the RACB trial design was sensitive and could therefore be better suited compared to the MGS model for the evaluation of reproductive traits. Reproductive traits were not statistically different over 4 generations in the MGS, but in differences between the groups became obvious in the 3rd and 4th litters of the RACB.

RNA microarrays and q-RT-PCR indicated differences between the groups. The findings were weak and need confirmation. However, a dietary impact on gene expression cannot be excluded. The high intra group variance could be due to different sensitivity of genotypes within the outbred mouse strain OF1 used in this study. For further investigation an RACB including several inbred strains could be useful. Some data obtained from the assessment of selected traits in organs by electron microscopy indicate a diet-host interaction that should be further evaluated.

The trial indicates that dietary interactions with the host organism have to be further evaluated. Regarding the sensitivity of the topic, studies are needed to extend the database using standardized feeding trials with clear endpoints such as reproductive performance and a backup by genomic, proteomic and metabolomic traits.

Summarising the study, the maize with the stacked event NK603 x MON810 affected the reproduction of mice in the RACB trial. Whether similar findings could be expected for other animals, needs to be evaluated in studies including reproductive traits. Future studies are necessary to determine the impact of normal and transgenic dietary ingredients on the organism.