

## Reversibility of hepatocyte nuclear modifications in mice fed on genetically modified soybean

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In the literature, the reports on the effects of a genetically modified (GM) diet are scanty and heterogeneous; in particular, no direct evidence has so far been reported that GM food may affect human or animal health.

Hepatocytes represent a suitable model for monitoring the effects of a GM diet, the liver potentially being a primary target. In a previous study, we demonstrated that some modifications occur in hepatocyte nuclei of mice fed on GM soybean. In order to elucidate whether such modifications can be reversed, in the present study, 3 months old mice fed on GM soybean since their weaning were submitted to a diet containing wild type soybean, for one month. In parallel, to investigate the influence of GM soybean on adult individuals, mice fed on wild type soybean were changed to a GM diet, for the same time. Using immunoelectron microscopy, we demonstrated that a one-month diet reversion can influence some nuclear features in adult mice, restoring typical characteristics of controls in GM-fed animals, and inducing in control mice modifications similar to those observed in animals fed on GM soybean from weaning. This suggests that the modifications related to GM soybean are potentially reversible, but also that some modifications are inducible in adult organisms in a short time.

Key words: cell nucleus, liver, genetically modified soybean, electron microscopy.

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In the literature, the reports on the effects of a genetically modified (GM) diet are scanty and heterogeneous (e.g., Schubbert *et al.*, 1998; Ewen and Pustzai, 1999; Chiter *et al.*, 2000; Edwards *et al.*, 2000); in particular, no direct evidence has so far been reported that GM food may affect human or animal health, and scarce are the studies on the effects of a diet containing significant amounts of GM plants (Malatesta *et al.*, 2002a,b, 2003; Vecchio *et al.*, 2004). These studies have been obviously focussed on organs and tissues which may be seen as potential targets (either directly or indirectly) of this diet. In this view, the liver is a primary site where the biotransformation of the products of digestion takes place through the degradation and/or detoxification of xenobiotic compounds received from the intestines or from the general circulation; in addition, the liver is involved in the synthesis of many plasmatic protein components and, more generally, in the overall metabolic control of the organism. Hepatocytes may, therefore, represent a suitable model for monitoring –at the cellular level– one of the targets of the diet. In a previous study (Malatesta *et al.*, 2002a), we demonstrated that some modifications occur in hepatocyte nuclei of mice fed on GM soybean: these changes are mainly related to the structural constituents involved in the transcription and splicing processes. In the present investigation, we aimed at elucidating whether such modifications can be reversed: to do this, mice fed on GM soybean from their weaning to the third month of age were submitted to a diet containing wild type soybean, for one month. In parallel, to investigate the influence of GM soybean on adult individuals, mice which had been fed on wild type soybean were administered for one month a GM diet. Morphometrical and immunocytochemical analyses have been carried out on hepatocyte nuclei at electron microscopy,

focussing on the nucleoplasmic and nucleolar constituents involved in the synthesis and maturation of RNAs.

## Materials and Methods

Pregnant Swiss mice were fed on a standard laboratory chow containing either 14% GM soybean (Padgett *et al.*, 1995) or wild type soybean. Twelve female mice (six from each experimental group) were grown on the parental diet from weaning until the third month of age. Then, the diets were reversed: the control group was fed on GM soybean (control-to-GM mice), while the GM-fed group was fed on wild type soybean (GM-to-control mice). All animals were fed on the reversed diets for one month and then killed by cervical dislocation. Liver samples were processed for electron microscopy by paraformaldehyde-fixation and LRWhite-embedding as previously described (Malatesta *et al.*, 2002a, 2003). Semithin sections were stained with 1% toluidine blue and observed in an Olympus BX51 light microscope. Ultrathin sections were stained with the EDTA method (Bernhard, 1969), for the visualization of ribonucleoprotein constituents. The samples were observed in a Philips CM 12 electron microscope operating at 80 kV.

Morphometrical analyses were performed both at light and electron microscopic level by using the computerised image analysis systems Olympus DP-Soft 3.0 for Windows 98 and Image Pro-Plus for Windows 95, respectively. Cellular and nuclear areas were measured at a fixed magnification (x100) on 30 hepatocytes per animal, then the nucleus/cytoplasm (N/C) ratio was calculated. Further morphometrical evaluations were made on electron micrographs (x39,000) of hepatocyte nuclei (10 micrographs/animal): nucleolar areas, percentages of fibrillar centres (FCs), dense fibrillar component (DFC) and granular component (GC) per nucleolus, FC area, index of nuclear shape

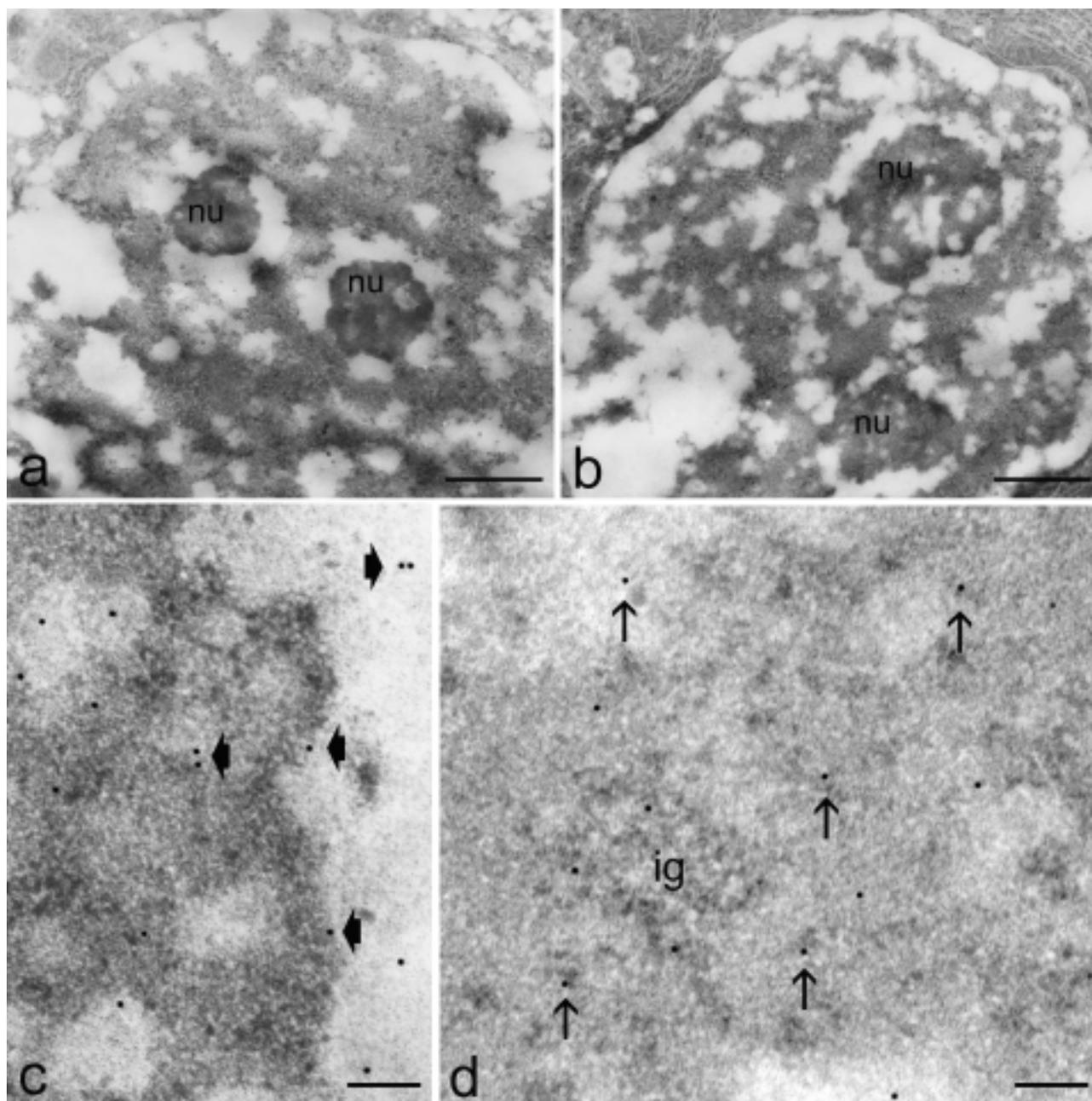
irregularity (the ratio between the measured perimeter and the circumference of the equivalent circle), perichromatin granule density (PG/ $\mu\text{m}^2$  of nucleoplasm) and nuclear pore frequency (NP/ $\mu\text{m}$  of perimeter) were considered (Malatesta *et al.*, 2002a, 2003). For immunocytochemistry, mouse monoclonal antibodies directed against polymerase II (Research Diagnostics Inc.), the non-snRNP splicing factor SC-35 (Sigma-Aldrich) and the nucleolar protein fibrillar (Cytoskeleton Inc.) were revealed by gold-conjugated antibodies (Jackson ImmunoResearch Laboratories Inc.) as described in Malatesta *et al.* (2003). The labelling density (gold grains/ $\mu\text{m}^2$ ) over cytoplasm, nucleoplasm and nucleolus was evaluated on 10 micrographs (x39,000) from each animal. For background evaluation, the labelling on the resin outside the tissue was considered. All statistical comparisons were performed by the Kruskal-Wallis one-way ANOVA test ( $p \leq 0.05$ ).

## Results and Discussion

The hepatocyte nuclei of all the animals considered in the present study generally showed a roundish shape, with clumps of condensed chromatin mainly distributed both at nuclear and nucleolar periphery (Figure 1a,b). In the nucleoplasm, no difference between the two animal groups was observed in the presence and distribution of perichromatin fibrils, perichromatin granules and interchromatin granules, structures involved in pre-mRNA transcription and splicing (Fakan, 2004). Conversely, the nucleoli showed different features; in fact, in control-to-GM mice they were characterised by many fibrillar centers (FCs) and abundant dense fibrillar component (DFC), whereas in GM-to-control animals they were more compact, with less FCs and prominent granular component (GC). The quantitative data (Table 1) confirmed that some nuclear features, such as the shape index,

**Table 1. Means $\pm$ SE values of variables considered in hepatocytes of the two animal groups. In each column, values identified by asterisks are not significantly different from one another.**

	Nuclear area ( $\mu\text{m}^2$ )	N/C ratio	Shape index	Pore frequency (pore nr/ $\mu\text{m}$ )	PG density (PG nr/ $\mu\text{m}^2$ )	Nucleolar area ( $\mu\text{m}^2$ )	FC area ( $\mu\text{m}^2$ )	% FC	%DFC	%GC
<b>Control-to-GM</b>	53.66 $\pm$ 1.19	0.21 $\pm$ 0.01*	1.09 $\pm$ 0.04*	0.45 $\pm$ 1.07*	1.13 $\pm$ 0.21*	1.02 $\pm$ 0.08*	0.03 $\pm$ 0.003*	6.25 $\pm$ 0.89	35.21 $\pm$ 1.71	56.25 $\pm$ 2.21
<b>GM-to-control</b>	45.01 $\pm$ 1.05	0.19 $\pm$ 0.01*	1.19 $\pm$ 0.06*	0.57 $\pm$ 0.09*	1.18 $\pm$ 0.28*	1.14 $\pm$ 0.06*	0.04 $\pm$ 0.008*	4.06 $\pm$ 1.49	29.74 $\pm$ 2.57	69.39 $\pm$ 3.92



**Figure 1.** a, b. Hepatocyte nuclei from a GM-to-control mouse (a) and from a control-to-GM mouse (b). The general aspect of the nucleus is quite similar, apart from the nucleoli (nu) which are more irregular and rich in small fibrillar centres in control-to-GM animals. Bars: 1  $\mu$ m. c. Immunolabelling with anti-polymerase II antibody; the signal is specifically located on pericromatin fibrils (arrowheads). d. Immunolabelling with anti-SC-35 antibody; the gold grains occur on perichromatin fibrils (arrows) and on intechromatin granules (ig). Bars: 0.2  $\mu$ m.

the pore frequency, the PG density and the nucleolar and FC area were similar in GM-to-control and in control-to-GM mice, while the nuclear area as well as the percentages of FC, DFC and GC were significantly different. Moreover, no quali-quantitative difference about the immunolabelling for polymerase II, SC-35 and fibrillar in was found between the two animal groups (Figure 1c,d and Table 2).

The comparison between these data and the previously reported hepatocyte nuclear features of mice fed on GM or wild type (control) soybean from weaning (Malatesta *et al.*, 2002a) reveals a complex situation. For example, the shape index and the pore frequency – both considered as indications of the metabolic rate, in relationship with the nucleocytoplasmic molecular trafficking – as

**Table 2. Mean±SE values of labelling densities obtained with anti-RNA polymerase II, anti-SC-35 and anti-fibrillarin antibodies on hepatocyte nuclei of the two animal groups. All value pairs are not significantly different from one another. Background: 0.22±0.05 gold grains/μm<sup>2</sup>.**

		Cytoplasm (gold grains/μm <sup>2</sup> )	Nucleoplasm (gold grains/μm <sup>2</sup> )	Nucleolus (gold grains/μm <sup>2</sup> )
<b>Polymerase II</b>	Control-to-GM	1.10±0.46	1.31±0.55	0.48±0.08
	GM-to-control	1.01±0.52	1.41±0.44	0.39±0.07
<b>SC-35</b>	Control-to-GM	1.87±0.08	3.42±0.19	0.27±0.08
	GM-to-control	1.98±0.14	2.81±0.15	0.31±0.09
<b>Fibrillarin</b>	Control-to-GM	1.89±0.48	1.58±0.85	7.69±1.16
	GM-to-control	1.74±0.18	2.01±0.64	8.32±2.21

well as the amount of transcription and splicing factors were previously found to be higher in GM fed-mice in comparison to controls. In this study, we have shown that these nuclear variables not only are similar in GM-to-control and in control-to-GM mice, but that their values are comparable to those previously reported in control mice. As for the nucleolus, it was previously reported that the percentage of DFC significantly increased in GM-fed mice, while the GC decreased, suggesting a higher nucleolar activity (Schwarzacher and Wachtler, 1993); interestingly, the GM-to-control mice show values similar to controls, while the control-to-GM group resembles the GM-fed mice. Finally, the nuclear area, which was found to be smaller in GM-fed mice than in controls, remains significantly lower in GM-to-control mice than in control-to-GM animals. It should be underlined that, in spite of these nuclear size modifications, no significant difference was found in N/C ratio, either in previous studies (Malatesta *et al.*, 2002a,b) or in the present work, indicating that the diet mostly affects the nuclear structural constituents.

In summary, our results demonstrate that a 1 month-diet reversion in adult mice can influence some nuclear features, restoring in GM-fed mice some characteristics typical of controls and inducing in control mice modifications similar to those observed in animals fed on GM soybean from weaning. This suggests that the modifications related to GM soybean are potentially reversible, but also that some modifications are inducible in adult organisms in a short time.

The multiple metabolic functions and, in particular, the central role in nutrient processing played by

the liver could explain such an immediate influence of GM food in hepatocyte nuclear features. At the present moment, we do not know which could be the factors present in the GM soybean capable of inducing such modifications. Among these, the possible presence in the chow of traces of glyphosate (Granby *et al.*, 2003), the herbicide to which the soybean used in our study has been rendered resistant (Padgett *et al.*, 1995), should also be taken in consideration in evaluating GM food effects on hepatocytes. Alternatively, it should be mentioned that soybeans are rich in phytoestrogens, non-steroidal compounds occurring naturally in many plants and able to bind to estrogen receptors, and that GM soybean has been reported to contain lower amounts of these molecules than wild type soybean (Lappé *et al.*, 1998). Taking into account the influence phytoestrogens exert at multiple levels on cell activity (Plessow *et al.*, 2003), in particular on hepatocytes (Cassidy, 2003), a difference in the daily intake of these compounds could explain the hepatocyte modifications in mice fed on GM soybean.

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