Retraction Notice

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History

Expression of Concern:

☐ yes, date: yyyy-mm-dd

☐ no

Correction:

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☐ no

Comment:

This paper involves in data fabrication so it does not meet the standard for publication.

This article has been retracted to straighten the academic record. In making this decision the Editorial Board follows COPE's Retraction Guidelines. Aim is to promote the circulation of scientific research by offering an ideal research publication platform with due consideration of internationally accepted standards on publication ethics. The Editorial Board would like to extend its sincere apologies for any inconvenience this retraction may have caused.

Editor guiding this retraction: Professor Alessandra Bordoni (EiC of FNS)

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Gamma-Glutamyl Transferase Activity in Kids Born from Goats Fed Genetically Modified Soybean

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ABSTRACT

The majority of animal feeding trials using GM feeds indicated no clinical effects while as concerns histopathological abnormalities in organs or tissues of exposed animals, the results are conflicting. Several data indicate liver and kidney problems as end points of GM diet effects. In rabbit fed GM soybean, it was hypothesised that cell metabolism of several enzymes was altered as well as elevated levels of LDH were revealed in tissues and organs of kids when mothers are fed GM soybean. The objective of this study was to investigate the fate of transgenic DNA and the activity of gamma-glutamyl transferase (GGT) in blood, liver and kidney from kids fed only milk of their mother fed conventional (control) or genetically modified soybean meal solvent extract (treated). PCR analysis revealed that fragments of multicopy chloroplast (trnL) and single soybean-specific (lectin) gene were found in samples of both groups. Fragments of transgenic gene were found only in treated kids: detection of 35S promoter was significant in liver, kidney and blood, and detection of CP4 epsps gene fragment was significant in liver and kidney. Concerning GGT, no differences were found in serum, while its activity was significantly ($P < 0.01$) higher in kidney (63.4 ± 7.8 vs 81.2 ± 11.3 u/g of tissue) and liver (40.1 ± 5.2 vs 62.6 ± 9.8 u/g of tissue) of kids from treated goats. The increase in GGT activity was confirmed by histochemistry.

Keywords: Genetically Modified Soybean; Goat; Kid; Gamma-Glutamyltransferase; DNA Fate; PCR

1. Introduction

Since the commercial release in 1996, genetically modified (GM) soybean has been increasingly used for livestock production even if concerns over safety of persist in the public, as regards either the detection of transgenic genes in animal systems or allergenicity and toxicity of GM plants. Roundup Ready® soybean is rendered tolerant to the glyphosate family of herbicides through expression of transgenic DNA from the CP4 strain of *Agrobacterium tumefaciens* that encodes 5-enolpyruvyl-shikimate-3-phosphate synthase (CP4 epsps). The majority of animal feeding trials using GM feeds indicated no clinical effects while as concerns histopathological abnormalities in organs or tissues of exposed animals, results are conflicting [1]. Several data indicate liver and kidney problems as end points of GM diet effects [2]. Tudisco *et al*. [3] hypothesised that cell metabolism of several enzymes was altered in rabbit fed GM soybean and the same authors found transgenic DNA fragments in goat milk but also in kids organs when mothers are fed GM soybean and revealed elevated levels of LDH in tissues, thus suggesting an increase in cell metabolism [4]. Aim of this paper was to investigate the presence of DNA fragments and the activity of gamma-glutamyltransferase (GGT) in blood and organs from kids whose mothers were fed GM soybean. GGT is a membrane bound enzyme widely distributed in mammalian tissues which are involved in absorption and secretion [5]. High levels of GGT are constitutively expressed mainly in the liver and serum levels have significance in medicine as a diagnostic marker [6,7].

2. Materials and Methods

2.1. Diets, Animals and Feeding

The trial was performed on 20 male kids born from two groups (control; treated) of goats fed solvent extract soybean (13% DM of a 18% CP/DM and 12.22 MJ/DM

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concentrate) which was conventional or RoundUp Ready®, respectively. Both groups of goats (10 animals each) received oat hay *ad libitum* and concentrate in amounts of 200 - 300 and 400 g/head/d, respectively 60 - 30 and 15 d before kidding. After kidding, concentrate was gradually increased up to 700 g/head/d and oat hay was replaced by alfalfa hay. Water was given *ad libitum*. The kids of both groups were allowed into individual cages positioned in a separate room, fed only mothers milk using a milk feeder and slaughtered at 60 ± 7 days of age.

2.2. Sampling

After 60 d from kidding, 100 mL of milk and blood samples (via jugular vein puncture) were collected from each goat. Blood was withdrawn at birth and before slaughtering from kids. Samples of liver and kidney (−20 g) from kids were removed, placed in separate labelled tubes and three aliquots from each sample were immediately stored at −20˚C in sterile tubes. Specimens from kidney and liver (5 g each) were washed in saline and stored at −80˚C to determine enzyme activity and in liquid nitrogen to perform GGT histochemistry.

2.3. DNA Analysis from Feed, Organ and Blood Samples

Conventional and GM soybean and milk samples were extracted according to the Wizard® Plus Minipreps DNA Purification System (Promega, Medison, Wis., USA) while organs and blood DNA using NucleoSpin® Tissue and NucleoSpin® Blood kit (Macherey-Nagel, Duren, Germany), respectively. All the extracts (in duplicate) were kept at –20˚C until further analysis. In addition, negative (buffer only) control to each set of DNA extraction was included [8]. DNA concentration and purity was determined by using a Biophotometer (Eppendorf, Hamburg, Germany) and PCR amplifications were performed on a Gene Amp PCR System 2400 (Applied Biosystems, Foster City, CA) and carried out as described previously [4]. The PCR was done 3 times, and samples with positive results at least twice were judged as positive [9]. In every PCR run, positive and negative controls were included to ensure reproducibility and absence of contaminants [10]. The amplification products were sequenced (in duplicate) using the ABI Prism model 310 capillary sequencer (Applied Biosystem, USA). Similarities with all sequences in the international nucleotide non-redundant data banks and with sequences from EST division were detected using the BLAST program [11] on network servers.

2.4. Enzyme Assay

The levels of gamma-glutamyltransferase (GGT) were determined in serum and homogenates prepared from samples of liver and kidney. Briefly, tissue (1 g) was homogenised in ice-cold homogenisation buffer (in mM): 280 mannitol, 10 KCl, 1 $MgCl_2$, 0.2 Pefabloc SC, 10 Hepes, pH = 7.0 adjusted to pH 7 with Tris-HCl 10 mM. After centrifugation at 10,000 × g for 10 minutes the upper layer was used for analysis. GGT activity was measured with a kinetic procedure by Spinreact Reagents (Spinreact, SA, St. Esteve de Bas, Girona, Spain) [100 mmol·L^{-1} Tris buffer (pH = 8.25), 100 mmol·L^{-1} glycylglycine, L-g-glutamyl-3-carboxy-p-nitroanilide as substrate] [12]. The absorbance at 405 nm was determined with a spectrophotometer (BIOMATE 6, Thermo Scientific Waltham, USA). Each sample was run in duplicate and the concentrations of GGT activity were converted into units/litre (serum) or units/gram of organs.

2.5. Histochemical Procedures

Samples of kidney and liver were taken and immediately frozen by immersion in liquid nitrogen (−170˚C) for 10 sec. Cryostate sections (4 - 8 µL) were mounted on slides, air-dried and incubated at 25˚C in the following freshly prepared solution: gamma-glutamyl-4-methoxy-2-naphthylamide (GMNA) (2.5 g·mL^{-1}), Tris buffer (0,1 M, pH = 7.4), saline solution (0.85%), glicylglycine, fast blue BBN (diazotized 4'-amino-2',5'-diethoxybenzanilide) (Sigma Chemical Company, ST Louis, MO, USA). This mixture was filtered prior to use since the stabilized diazonium salt was not completely soluble. The final concentration of substrate into incubation medium was 0.125 mg·mL^{-1}. A stock substrate solution was prepared by dissolving 25 mg GMNA in 0.25 dimethylsulfoxide and 0.5 mL NaOH (1N) and then adding 9 mL distilled water [13]. This solution was stable for 3 days at 4˚C. Following incubation, the sections were rinsed in saline (0.85%) for 2 minutes and then transferred to a 0.1 M solution of cupric sulfate for 2 minutes. After another saline rinse, the sections were rinsed in distilled water, dried and mounted in PBS-glycerol (1:1). The nuclei were counterstained with haematoxylin coloration. A negative control was prepared for liver and kidney. Five slides for liver and kidney of both the groups were independently evaluated by two observers by using a Leica DMRA2 microscope.

2.6. Statistics

The presence of plant DNA fragments in kids blood, liver and kidney was analysed by using the Chi-square test vs those detected in milk from the corresponding mothers at day 60 (slaughtering time). Results for enzyme assays were expressed as mean ± standard deviation. Differences within groups were calculated by one-way ANOVA [14].

3. Results

3.1. DNA Analysis

The quality of animal DNA samples was verified using

primers that were used to amplify a conserved portion of caprine mtDNA 12S rRNA sequence [4]. In conventional and GM soybean, specific amplicons of chloroplast (*trnL*, 100 bp) and lectin (118 bp) genes were amplified while only GM soybean was positive for 35S promoter (195 bp) and CP4 epsps (145 bp) fragments. Chloroplast DNA and lectin gene fragments were found in the majority of control and treated samples either from goats or kids. By contrast, 35S promoter and CP4 EPSPS fragments were amplified only in samples from treated group (**Figure 1**). The Chi-square analysis revealed that, in blood, liver and kidney, significant results were detected for chloroplastic and lectin fragments ($P < 0.01$ in both groups) and 35S promoter ($P < 0.01$ in both groups) while that of CP4 epsps gene fragment ($P < 0.05$) from treated group (**Table 1**).

3.2. Gamma-Glutamyltransferase Activity

Significant differences ($P < 0.01$) in GGT activity were found in kids kidney (63.4 ± 7.8 vs 81.2 ± 11.3 u/g of tissue) and liver (40.1 ± 5.2 vs 62.6 ± 9.8 u/g of tissue). Kids from treated goats always showed higher levels of the enzyme. No statistical differences were found in serum. The increase in GGT activity was confirmed by histochemistry (**Figure 2**). GGT histochemistry showed a widespread distribution of enzyme activity in the tissues that were examined. The GGT reactivity was expressed in the cytoplasm of hepatocytes as red-purple coloured diffuse and fine granules. In tissue sections used as negative controls this staining was light red-purple, epithelial cells of renal tubules and hepatocytes all expressed GGT activity. GGT staining was more intense in hepatocytes and epithelial cells of renal tubules of treated than in those of control animals. In each panel, lane M contains a 100-bp DNA ladder, "–" is a negative control (no DNA template), and "+" is a positive control (DNA extracted from Roundup Ready soybean meal).

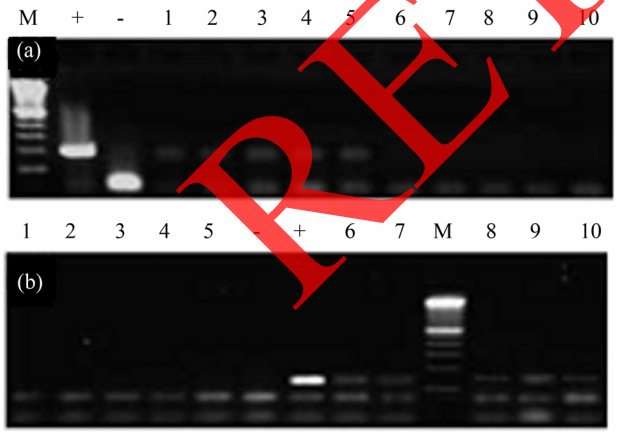

Figure 1. Representative data of amplified transgenic DNA fragments. (a) 35S promoter fragments (195 bp) in milk and blood from control (lines 6 and 7, respectively) and treated (lines 1 and 2, respectively) goats; in blood, kidney and liver from control (lines 8, 9, 10, respectively) and treated (lines 3, 4, 5, respectively) kids; (b) fragments of CP4 EPSPS gene (145 bp) in milk and blood from control (lines 1 and 2, respectively) and treated (lines 6 and 7, respectively) goats; in blood, kidney and liver of control (lines 3, 4, 5, respectively) and treated (lines 8, 9, 10, respectively) kids.

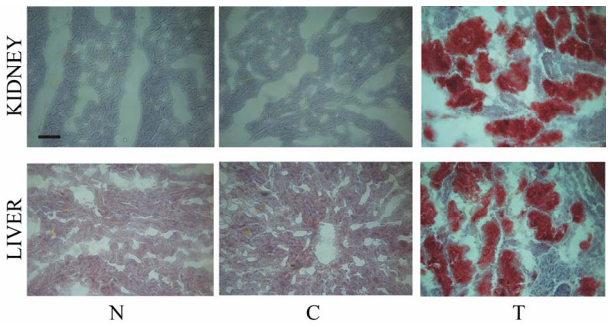

Figure 2. GGT histochemistry in kids liver and kidney on negative control sections (N), control (C) and treated (T) animals. GGT activity was revealed by a red-purple staining. The intensity of GGT staining was higher than in the same tissues of control animals. Bar = 50 µm.

Table 1. Number of dams producing milk at 60 days in lactation in which DNA sequences was detected. Number of kids with organs in which DNA sequences was detected.

	Control				Treated			
	Milk	Kids			Milk	Kids		
	Day 60	Blood	Kidney	Liver	Day 60	Blood	Kidney	Liver
Chlor	9	9*	8**	8**	9	8**	8**	8**
Lectin	7	7**	6**	6**	8	8**	7**	7**
35S	-	-	-	-	7	6**	6**	6**
CP4 epsps	-	-	-	-	6	5*	4*	4*

Milk derived from dams of treated (n: 10) or control (n: 10) group. The lectin, 35S and CP4 EPSPS fragments were investigated only in those samples, which were positive for the chloroplast DNA fragment (Chlor). *P, 0.05; **P, 0.01. This P-value indicates a significant proportion of the kids found positive for the same DNA fragments detected in milk.

4. Discussion

Transgenic plant DNA has been found by other authors [15-19] in organs and tissues from animals fed GM feeds. On the contrary, in trials carried out on dairy cows fed GM soybean [20], the chloroplast DNA fragments were detected in milk but no transgenic fragments were found. In agreement with previous studies [3,21] our findings confirm the likelihood that plant DNA fragments can survive digestive processes, and that they can be transferred to blood and milk [4]. In addition, the detection of plant DNA in blood and organs of kids fed only mother

milk could support the hypothesis of a gene transfer through milk. Our results suggest that the detection of single copy genes in kids were not a result of environmental contamination during collection, as well as the negative results in samples from the control group confirmed the absence of transgenic fragments.

In kids serum the levels of the enzyme did not show significant differences, thus suggesting that no adverse effects were induced by GM feed in treated animals, as previously reported in dairy cows [22]. By contrast, the increase of the enzyme in cells from both organs suggests some change in cell metabolism that leads to a higher synthesis. The meaning of such increase of GGT expression in treated kids organs is not clear, many substances, including drugs, carcinogens and alcohol have been shown to increase GGT gene expression in several cells and tissues [23,24]. On the other hand, increased expression of GGT during oxidative stress facilitates GSH turnover, de novo GSH synthesis and metabolism and detoxification of GSH conjugates that increase cell resistance to stress. Therefore, regulation of GGT gene expression is an important adaptive response to protect cells and tissues from oxidative injury. In any event, besides the possible implications of a GGT increase at cellular level, it is a fact that the expression of the enzyme changed in animals fed GM soybean.

5. Conclusion

Our results show that transgenic genes could be detected in organs and blood of kids whose mothers were fed GM soybean. In these animals, the presence of normal serum levels of tested enzymes shows that no cell injury occurred but cell metabolism seems to be affected both in liver and kidney. Such results are in agreement with recent studies which showed alteration of other enzymes local production and confirm the feeling that research concerning the effects of GM feeding is still far from over.

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