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Evaluación histológica y de expresión de genes del páncreas de ratas alimentadas con soya transgénica

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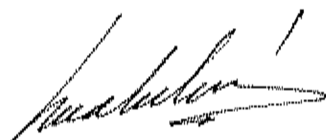

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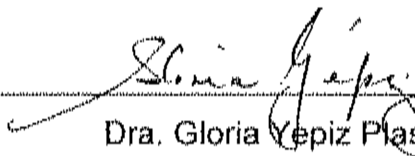

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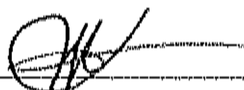

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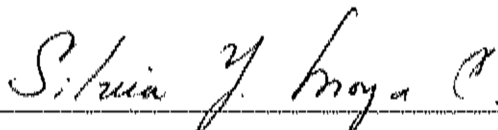

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Sonora

No puedo escribir de tí
más de lo que ya está escrito,
mis palabras son viejas, huelen a naftalina:
Ojos negros, tierra consentida.
Mujeres hermosas,
Atardeceres de ensueño.
Eres sonora Sonora.
Me has marcado como hierro encendido
y me quemaste hasta mis entrañas.
Empezaste en la piel,
terminaste en mi corazón.
No puedo escribir más de tí,
porque vienen los recuerdos,
y lágrimas se agolpan en mí,
y mi mentón se aprieta.
Eres libro abierto, que contagia sabiduría.
Eres piel que se estremece en noches de frío.
Eres calor que abraza, que derrite mi alma.
Tu mar son lágrimas
de los que se van y que te extrañan.
Eres sonora Sonora,
me voy de tí, maldita sea la hora.

Con cariño, JMG

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SINOPSIS

Las técnicas de la ingeniería genética, aplicadas a las plantas, permiten generar nuevas variedades con características que cubren o resuelven problemáticas específicas. Tales son, la resistencia a la exposición a herbicidas, condiciones ambientales desfavorables e incluso la expresión o sobreexpresión de nutrimentos importantes en la dieta humana. La gran diferencia con las técnicas tradicionales no sólo es su rapidez, sino que hacen posible introducir genes de cualquier otro organismo, ya que no se limitan a las especies taxonómicamente relacionadas. Así, se entiende por planta modificada genéticamente (GM, por las siglas en inglés) aquella que ha sufrido alguna manipulación de su material genético, para expresar un gen que le confiera una característica deseada. Si el origen de tal gen es un organismo ajeno a la planta misma, puede aplicársele el término de planta transgénica al producto final. Cuando la nueva característica se obtiene modificando la expresión de un gen propio, la planta sólo recibe el término de modificada genéticamente.

Algunos ejemplos de plantas transgénicas son los cultivares resistentes a herbicidas como el glifosato y glufosinato, a plagas, y a condiciones climáticas adversas como sequía y salinidad. También, están aquellos que aportan beneficios directos al consumidor, debido a su mejor valor nutricio, como el arroz dorado con un contenido alto de carotenos, Los productos de los cultivares GM, se utilizan en la misma forma que sus contrapartes convencionales, como alimentos completos o como ingredientes. Por extensión, a dichos productos se les llama alimentos transgénlcos o modificados genéticamente según el caso.

Debido al amplio rango de aplicaciones de los cultivos modificados genéticamente y a las ventajas potenciales que ofrecen tanto a los productores como a los consumidores, sería de esperarse una buena aceptación por parte de los consumidores. Sin embargo, en su corta historia de poco más de 10 años, han generado y siguen siendo un tema controvertido, al que alimentan continuamente los medios de comunicación masiva.

continuamente los medios de comunicación ,rn siva. El fiel que equilibre la discusión entre la aceptación y r1l rechazo, debe ser la lnv(:,stlgación cienHfica. Se espera su pa11icipación activa en el proceso de evaluación de riesgos de los organisnws GM en general y de alimentos GM en particular. Estos riesgos incluyen aquelk.ls tanto para el medio ambiente y la biodiversidad como para la seguridad alimentaria, nutrición y salud humanas.

En la práctica, el proceso de evaluación tie los riesgos del consumo de alim(1ntos GM para la nutrición y salud humana, no ha sido sistemático. Los principios del análisis de riesgos se desarrollaron y usan para abordar peligros químicos, ,nicrobiológicos y factores nutricios, no para analizar alimentos compk,t.os. Aun asl, se pueden aplicar en términos g,rnEnales a los alimentos GM, con adecuaciones y modificaciones.

Actualmente, los alimentos nuevr)s se someten al proceso de análisis de riesgos del Codr x Alirmmtarius, comisión creada por la FAO/OMS para desarrollar normas que protejan la salud de los consumidores. Sin embargo, al principio no fue asi, sino que cada alimento GM fue evaluado usando diferentes periodos de alimentación, diw1rsos rnr)delos animales y las variables medidas no r ran comparables entre uno y otro estudio. A pesar de esto, en todos los estudios ha sido una constante el no encontrar efectos adversos en el estado nutricio de los organismos de prueba, pero si efectos a niveles microscópicos y moleculares en diferentes órf¡anos y tejidos. Por lo tanto, hoy dla, no hay conclusiones definitivas ac;erca de la inocuidad de los alimentos GM.

La soya tolerante al glifosato (GTS, por sus siglas en inglés) 40·3·2 es en la actualidad el principal cultivo transgénico. Éste, ocupó el 57 % del área total sembrada con cultivos GM en el 2006, A este cultivar se le confirió la resistencia al herbicida glifosato, por medio de la inserción del gen codificante de la enzima 5--enolpiruvilshikimato-3-fosfato sintasa (E.PSPS) de Agrobacter/um sp. cepa CP4. Las evaluaciones iniciales de seguridad del consumo de soya transgénica encontraron que no habla reaGcioties de toxicidad aguda del'ivt das de la EPSPS-CP4, que su calidad nutricia (l'a comparable a la convencional y que no afectaba al organismo que la consurnia. Sin embargo, r:istudios posteriores que

usaron indicadores d(respuesta mt1s sensibles, encontraron que (JI ctmsurno de soya GM afectaba el metabolismo de ór¡Janos como !ligado, ri1'1ones, testículos y pefmcreas. En estudios preliminares sobre los efectos del consumo de la soya GM en ratas Wistar a nivel de expresión de genes en pl'.mcreas, se obtuvieron resultados que sugieren dalio pancreático con posterior adaptación (Magafia·Gómez, 2003).

Lf¡ protelna de soya, independientemente si es transgénica o no, es rnuy utilizada en la industria alimentaria. Se emplea corno ingrediente en productos cárnicos emulsificados, bebidas, productos de panificación, fórmulas de fllirnentación especial y la mayorla de los alimentos industrializados.

La presente tesis, se justifica por el uso tan extendido de la protelrn de soya corno ingrediente alimentario; por la evaluación tan somera para liberar al mercado la soya evento GTS 40-3·2: así como por la controversia en la seguridad del consumo de transgénicos. Las premisas, se basaron en los resultados previos de la evaluación de la protelna, propios y de otros autores. La hipótesis propuesta fue que el consumo de proteína de soya transgénica induce efectos adversos en el páncreas de ratas, tanto a nivel histológico co1110 en la expresión de genes, con adaptación al corto plazo. Asl, el objetivo fue evaluar el efecto del consumo de aislado prnteico de soya resisterite a glifosato, en la histologla del páncreas y en la t,xpresión genética de tripsinógenos y de la protelna asociada a pancreatitis (PAF'), en ratas Wistar recién destetadas.

Para contrastar la hipótesis, i;e evaluaron los cambios histológicos en páncreas y en la expresión genética dE! tripslnógenos y PAP mediante PCH en tiempo real en un modelo animal de consumo de aislado proteico de soya transgénica o de soya convencional. Previarnentt1 se caracterizó el ADN recornbinante de las fm ntes proteicas a evaluar, para verificar que se tratara de soya transgénica. Los resultados del bioensayo fueron comparados con los obtenidos en un modelo ani111al de pancrc,atitis aguda inducida con cerulelna,

El desarrollo de los estudios para cumplir el objetivo propuesto, se encuentra descrito <1n los diferentes capltulos que conforman esta tesis. El primer capitulo es una revisión del r stado del a1te actualizada a julio del 2007,

<311el que se resaltan aspectos con,o la controversirl de los estudios *in vivo* para evaluar posibles efectos derivados del consumo de alilrn ntos transgénicos. Asl rnismo, se hace patente la necesidad de llevar a cabo estudios con indicadores más sensibles de efectos en kl salud.

El segundo capitulo de la \(sis es un articulo ya publicado que describe la replicación de una pancre,1titis aguda en ratas, por 1'1edlo de la administración de cerulelna, un análogo de la colecistoclnina, cuyo (fr1cto fue una sobreestimulación del páncrer s. En este modelo se estudiaron los cambios histolót icos y en la acumulación del ARN mensajero (ARNrn) de una prott:l'lna asociada a pancreatltis (PAP) desde la fase aguda hasta la recuperación. El am lisis histológico reveló que desde las 9 h post,lnducción hubo una infiltración de linfocitos polirnor1'onuclearns y formación de vacuolas citoplasmát.icas con una recuperación a valores similares a los controles, a los 5 dlas. También se observó un aumento progresivo del d(sorden acimu y de la depleción de zlmógenos con un pico máximo en el d la 15 y retorno a los valores normales a los 30 dlas. La acumulación del ARNm de F'AP fue mayor¡ las 24 h y regresó a niveles control a par1it' de los 5 días post-Inducción, Este estudió reveló que durante el curso de la pancreatltls ocurre una regeneración y rediferenciación di;il tejido pancreático y confirmó que PAP es una proteína de resput sta aguda en lt pancreatitis,

Por último, el tercer capitulo de la tesis contiem, el desarrollo del bioensayo para evaluar los efectos del consumo de protelna de soya transgénica tn el páncreas de ratas, comparándolos con los de un grupo alimentado con un aislado de soya no transgénica. La calidad nutricia de las dietas, evaluada según la eficiencia proteica y alimentaria, fue comparable entre ambos grupos de anlmal(S. Asl también fue comparable el contenido de inhibidores de tripsina y quimotripslna entre ambas fuentes de protelna de soya. Hubo diferencias en cuanto al contenido de isoflavonas, pero en ambos casos se encontraron entre los limites normales para este tipo de producto, Corno en el caso del modelo de pancreatitis, se hici(ron estudios histológicos del páncreas de las ratas y se t?Valuó la r xpresión genética en dicho órgano. El

1málisis histológico de los páncrc-:¡as de ratas alimentadas con soya GM mostró un desorden adnar y depleGión de gránulos de zimógeno en forma similar a como ocurrió en los animales con pancreatitis inducida. Asl mismo, en el grupo GM se observó una mayor acumulación de ARNm de PAP entre el dla ·1 y 3, corno ocurrió en pancreatitis y un aumento del ARNm de tripsinógenos en los dlas 1 *y* 15 del estudio.

La integración de los resultados obtenidos en esta tesis permitió concluir que la ingestión de la protelna de soya transgénica afectó la función pancreática. El efecto consistió en una respuesta aguda del páncreas mediante la acumulación del Af"!Nm de PAP *y* cambios histológicos asociados a procesos de rediferenciación de las células acinams, tiasta una recuperación completa en un periodo de 30 dlas.

CAPÍTULO 1

Are genetically modified crops a risk to human nutrition and health? A review

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Are genetically modified crops a risk to human nutrition and health? A review

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Abstract

Foods produced through genetic engineering, including such staples as soybean, corn, canola, and potatoes, have already reached the consumer market place. However, there is still a debate about their security for human health and environment. Currently, official standards for food safety assessment have been published by the *Codex Alimentarius* Commission of FAO/WHO but despite the existing guidelines, published studies on risk assessment have not followed a defined pattern. Albeit, neither difference in nutritional performance nor in growth parameters has been found, microscopic and molecular changes in different organs and tissues have been reported. Much more scientific effort is necessary in order to obtain confidence for acceptation of genetically modified foods. New proposals based in experimental data for analysis before the introduction of GM foods to the marked, are needed.

Key words: genetically modified crops, in vivo studies, risk assessment.

Genetically modified crops: An overview

Foods produced through genetic engineering, including such staples as soybean, corn, canola, and potatoes, have already reached the consumer marketplace. This technology aims to express novel and desirable traits, offering some advantages for producers or consumers, over conventional crops. The results are organisms called genetically engineered or genetically modified (GM).

The importance of biotech crops is evident since the global planted area has increased by more than sixty-fold from 1.7 million hectares in six countries in 1996 to 102 million hectares in 22 countries in 2006. The world's top six producers –the United States, Argentina, Brazil, Canada, India and China – account for more than 90% of global GM production, more than 50% just in the United States. The genetically modified soybean was still the main biotech crop in 2006, occupying 57% of global biotech area, followed by maize (25%), cotton (13%) and canola (5%). [1]

In spite of the various genetic modifications, the GMs are currently classified in two generations, according to who receives the benefit. The first one refers to biotechnologically derived seeds, which offer farmer benefits such as lower chemical input and reduced farming costs. Such seeds present specific pest, disease or virus resistances. Examples of the first generation GM crops are the herbicide resistant (glyphosate) soybean, [2] insect resistant maize [3] and herbicide and insect resistant potato. [4] These crops are nowadays planted on tens of millions hectares of farmland. [1]

The second generation of GMs consists of crops consumer-friendly. This offer to the processor, end-user and consumer specific benefits such as increased levels of protein, modified and healthier fats, modified carbohydrates, improved flavor characteristics or increased levels of desired phytochemicals. Examples are rice with higher iron and zinc levels; [5-7] tomato with carotenoid, flavonoid and phenolics enhanced levels; [8-10] maize with augmented vitamin C

levels; [11] improved amino acid composition soybeans [12] or enhanced calcium content potato. [13]

Understanding the controversy

Since the apparition of GM crops, society, legislators, governors and academics have been involved in discussions about different issues. The advantages of these crops to developing countries, economic issues, environmental impact, ethical and social considerations, public confidence in regulatory regimes and others, are recurrent topics. However, the most frequent issue in current debate of GM is whether these are secure for health and environment, in part because they are constructed in forms that may appear dangerous. However, the concern is rather about possible consequences of this technology than on the technology itself. Although environmental risks are not discussed any further in this work, since this review is focus on health effects of GM products, excellent reviews can be consulted. [14-19]

Production and commercialization of GM crops in United States, United Kingdom and other countries was not a problem until summer of 1998, when the controversy started. Cheese elaborated with enzymes from GM microorganisms, tomato paste from GM tomatoes with increased shelf life, and processed foods containing herbicide- or insect-resistant soybean and maize were sold at marketplaces. The tomato paste had large labels advertising the fact that it was made from genetically modified tomatoes and no hostility appeared towards the GM products. The labeling allowed consumers to choose between GM and traditional varieties. At the same time, academic and government scientists were actively performing experiments and publishing research on plant transformation and its bio-safety.

In 1998, when Dr. Pusztai appeared on UK television announcing that GM potatoes, with transgenic snowdrop lectin were toxic to rats and affected their immune system [20] media attention was focused on GM crops and it was the catalyst of the reaction against such crops and foodstuffs. Soon after, detection

of GM soybean in manufactured foods, without appropriate labeling, accented the public fear in the UK where the "mad cow disease" crisis in 1996 had sensitized the population. People were able to relate some diseases with foods and were alerted about food safety. Since then different facts related to GM foods and health, have been disclosed by the media and nowadays there is a huge press and public concern about the health safety of GM crops, and it is not clear yet how is going to end.

Risk assessment of genetically modified foods

In the GM foods issue, the science should underlie health safety. After the introduction of recDNA technology in plant breeding and biotechnological food production systems, it has been necessary to define internationally harmonized guidelines for the safety assessment of foods derived from GM crops. At an early stage, first reports on the issue placed the bases for later safety strategies. [21] Since then, guidelines have been improved to obtain broad international consensus among experts on food safety evaluation. Currently, official standards for food safety assessment have been published by the *Codex Alimentarius* Commission of FAO/WHO. [22] Despite the existing guidelines, published studies on risk assessment have not followed a defined pattern. One view is that the safety assessments of these foods are not as rigorous as those for new chemicals or drugs.

In a review, Pelletier [23] shows that principles of food safety assessment were originally made to guide regulations related to the introduction of GM microbes and plants into the environment, but no attention was given to food safety concerns in these reports. However, these principles have been widely cited as authoritative scientific support for approach to food safety assessment. Furthermore, the Statement of Policy of the FDA, acknowledging the GM foods as presumptive GRAS (generally recognized as safe) was made under critical gaps in scientific knowledge concerning the compositional effects of genetic transformation and severe limitations in methods for safety testing. [21, 23]

Another pitfall in the food safety assessment is the concept of substantial equivalence. Initially it was formulated by OECD (1993), based on the idea that existing foods can serve as a base for comparing the properties of a GM food with its conventional counterpart. A Joint FAO/WHO Expert Consultation on Biotechnology and Food Safety concluded that: "When substantial equivalence is established for an organism or food product, it is regarded to be as safe as its conventional counterpart and no further safety consideration is needed." However, this conclusion is not clear since it does not sets if the new variety should be compared with the parental variety grown under same conditions or with the range of values of all untransformed varieties grown under varying conditions. Moreover, a chemical composition analysis is not enough to conclude on the allergological and toxicological safety of GM crops and foods. Such approach ignores the possible presence or increased accumulation of new allergens or toxins as a result of non-intended secondary effects from the modification. Effective techniques for metabolic and proteomic profiling are necessary, but they were not available when GM commercial production started and these techniques are not yet widely applied for this purpose.

The substantial equivalence is established by the determination of the phenotypic characteristics and the complete chemical composition of the plant including nutrients, toxicants, antinutrients, and allergens. However, it has never been properly defined; it is not clear when the degree of difference between a conventional food and its "substance" ceases to be acceptably "equivalent". Therefore, this vagueness makes the concept unacceptable to the consumer. Nowadays, it is accepted that the substantial equivalence is not a safety assessment *per se*, it just helps to find similarities and differences between conventional and GM food, but it is just the beginning and further investigation is needed. Guidelines have been prepared by ILSI Europe [24] and FAO/WHO [25] recommending that the safety evaluation should be on the concept of substantial equivalence, considering additional parameters. For instance, molecular characterization, expressed substances (non-nucleic acid substances),

compositional analyses of key components, evaluation of metabolites, food processing, nutritional modification and toxicants.

A main gap in the risk assessment of GM foods was the assumption that unintended consequences appear no more likely in GM than in conventional foods. This guiding principle was justified assuming that genetic engineering is an extension of traditional plant breeding methods and unintended changes are possible with all breeding methods. [21] However, some studies confirm that genetic engineering can increase the levels of metabolites other than those directly related to the transgene. [26]

The possible health consequences of GM foods have been considered in some reviews. [27-29] In all of them the necessity of more scientific effort and investigation to assure that eating GM foods in the long term is not likely to provoke any form of health problems was strength. Moreover, there is consensus that adequately tests of GM products, case by case, is necessary before to introduce them into the market, together with a tendency towards the use of more sensitive indicators. Albeit, does not seem to be yet a conclusion on the safety of GMs and their effects in human nutrition and health, the contribution of science is undeniable and in the light of new knowledge, early results should be reconsidered.

Study Case 1: GM soybean

The glyphosate tolerant soybean (GTS) event 40-3-2 (Roundup Ready™) has been widely studied; however, it is continuously generating controversy. Earlier it was demonstrated that there were no differences in composition between GTS and its isogenic line. [30] Also, no toxicity derived from the novel expressed protein (5-Enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* sp. strain CP4, EPSPS-CP4), [31] and finally, the feeding value was not altered by genetic modification. [32] However, it should be considered that insecticide unsprayed Roundup Ready™ (RR) soybeans were used in the compositional study [30] and no data had been published on the composition of

genetically modified soybeans sprayed with glyphosate in the normal course of production. Additionally, the tested EPSPS-CP4 protein for toxicity was purified from bacteria not from soybean and differences could exist.

Regarding to GTS, it is relevant to mention that genetic construct introduced into soybeans was described, but no information had been available about the exact end-points of the insert DNA and the sequences following those end-points. It was demonstrated that integration of the insert DNA produced several rearrangements at the 3' NOS junction and that the genomic plant DNA at the pre-integration site may have been rearranged. [33] A further 250-bp fragment of the *epsps* gene localized downstream of the NOS terminator is further processed, resulting in four different RNA variants which might code for (as yet unknown) EPSPS fusion proteins. [34]

The health risk assessment of GM soy intake has been mainly based on questions related to the nutritional performance. In several studies [32, 35-37] differences neither in growth parameters nor organ weight or its appearance have been found. In spite of this, adverse effects were detected at ultramicroscopic and molecular levels. The effects of the chronic ingestion of RR soybean were studied in Swiss mice. [37-39] Pregnant mice were fed on diet containing 14% GM or wild soybean. Twelve respective litters were used for feeding on the parental diet. The mice were weighed and then sacrificed at 1, 2, 5, or 8 months old. Interestingly, body weight of mice and macroscopic modifications of pancreas and liver were found without significant differences between control and GM soybean-fed animals. Nevertheless, when the analysis was directed towards ultrastructural morphometrical and immunocytochemical levels, differences were found. In liver, GM fed-mice showed irregularly shaped nuclei, suggesting to the authors a high metabolic rate, higher number of nuclear pores, which mean an intense molecular trafficking. Similarly, the nucleoli showed typical signs of increased metabolic rate. In pancreas, although no structural modification occurred in acinar cells, such differences were marked. The zymogen content, total zymogen area, percentage of cytoplasmic area occupied by zymogen and zymogen granule size were always smaller in GM

soybean-fed than in control mice. So it seems that the diet containing significant amounts of RR soybean influences the zymogen synthesis and processing. Analysis of mice testes suggested that, during the 2-8 months interval, a transient transcriptional decrease occurs in mice fed on the GM diet. [40] Although a possible role played by traces of the herbicide to which the soybean is resistant is discussed, the reason leading to these modifications is not clear and further investigation is suggested.

GM soybean was tested for metabolic effects in rabbits. [41] The animals received a diet with 20% soybean meal from conventional or RR soybeans (it represents around 65% of total protein, a significant amount of soy protein) for 40 ± 5 days. The protein level of the diets was according to nutrient requirements for rabbits. [42] No effects were detected on body and organ weights, but a significant increase of lactic dehydrogenase, mainly the LDH1 isoenzyme was found in kidney and heart, suggesting a potential alteration in the local production of the enzyme. The authors, [38] suggest increased cellular metabolism. The results lead to suggest an accurate enzymatic analysis as additional tool to evaluate the risks of GM feeding on cell metabolism even in absence of clinical and biochemical signs.

GTS also has been evaluated in the Atlantic salmon *Salmo salar* L. The results were published in two peer-reviewed papers. [36, 43] For 3 months post-smolt salmons were fed on diet holding 130 g kg^{-1} of protein from soybean. Interestingly, a GM diet was compared with a standard counterpart (commercial hybrid, not isogenic line) non-GM diet and again compared with a standard fish-meal diet without soybean protein. Although this can be criticized, the comparison of the products is relevant, since the consumer purchases them from the market. A pitfall in the design of diets was the slight difference in levels of anti-nutrients between the three diets. In spite of this and similar to other evaluations, no significant differences in feed utilization, whole body, and liver and muscle fatty acids profiles were measured. The relative sizes of organs like kidney, liver and brain were similar in all dietary groups and larger in the fish fed with soy. The spleen was the only organ that varied significantly in size, being

bigger in the fish fed GM-soy. Fish fed the soy diets of either type had reduced distal intestine. Incidence of moderate inflammation was higher in the GM-soy group. Furthermore, head kidney lysozyme was higher in GM compared with non-GM soy fed fish, a possible indicator of phagocyte activity or its presence in the tissue.

Brake and Evenson, [35] tested the potential toxic effects of RR soybean using mouse testes. Pregnant mice were fed a GTS or a non-transgenic diet during gestation and lactation. After weaning, male litters were maintained on the respective parental diets. At 8, 16, 26, 32, 63, and 87 days after birth, the testes were surgically removed, and the percentage of germ cell populations was measured by flow cytometry. The results showed no apparent differences between the mice fed the GTS diet and those fed the conventional diet. It was concluded that GTS had no measurable or observable effect on fetal, postnatal, pubertal, or adult testicular development or body growth.

The absence of differences in gross indicators by GM soybean intake, across different studies is constant. Although different experimental conditions do not permit inferences, a tendency towards molecular changes could be observed. Molecular changes are suggestive of some kind of cell damage and immune response. Early studies must be seen as the support for new experiments. In the way, obstacles as the difficulty to acquire the non-GM parent line of the GM-crop used in the study, the nutritional equivalence between diets and the best animal model must be solved. It is clear that health security of GM soybean intake have been not proved yet.

Study ***Case 2: GM corn***

Although GM corn follows the GM soybean as the second main biotech crop in 2006, [1] it is the crop with more genetic modifications. Several studies testing its safety have been published. Some studies are not true risks assessment. Authors test more agronomic features than biological. In some

cases, it is concluded that GM crop is substantially equivalent to its conventional counterpart. Examples of these studies are mentioned below.

Barriere et al., [44] evaluated the feeding value of GM Bt176 corn hybrid (Rh208Bt) respect to its isogenic line (Rh208) in three separate feeding trials during 1, 13 and 3 week respectively with Texel sheeps and Holstein cows. Variables measured like organic matter digestibility, crude fiber digestibility, neutral detergent fiber digestibility or coagulation properties of milk were not significantly different among Rh208 and Rh208Bt hybrids. Examinations of nutritional status were not given. The authors concluded that cattle could be fed equally well with a conventional or a GM Bt176 corn silage.

Donkin et al., [45] tested the effects of feeding silage and grain from corn resistant to European corn borer (Bt-MON810, experiment 1 and 2) and glyphosate-tolerant Roundup Ready corn (RR-GA21, experiment 3) on feed intake, ruminal digestion, and milk production in dairy cattle. Diets contained 42 to 60 % corn silage and 20 to 34 % corn grain from Bt-MON810, RR-GA21, or the appropriate non-transgenic counterpart. Treatments were applied using a switchback design of three periods of 21 d in experiment 1 and 28 d for experiment 2 and 3. Differences in the variables studied were not found, demonstrating equivalence of nutritional value and production efficiency for Bt-MON810 and RR-GA21 corn. Although they had suitable levels of protein, the one of corn is limited.

Erickson et al., [46] carried out three experiments testing the effect of Roundup-Ready events GA21 or nk603 corn on steer performance and carcass characteristics. The assay periods were 92, 94 and 144 days, including a 20-d diet adjustment period in experiments 1 and 2 and 28 d for the third. The final diets contained a maximum of 75% of maize. Since performance and carcass characteristics were not affected, the authors concluded that Roundup Ready corn is similar to the non-transgenic when fed to finishing feedlot cattle. There were no additional examinations of organs or biochemical indicators of the nutritional status of the animals.

Hammond et al., [47] presented the results of a 13-week feeding study in rats with grain from RR corn (tolerant to the herbicide glyphosate). Diets were formulated with 11 and 33% (w/w) of RR corn and same level with non-transgenic grain (controls). Overall health, body weight, food consumption, clinical pathology parameters (hematology, blood chemistry, urinalysis), organ weights, gross and microscopic appearance of tissues were comparable between groups fed diets containing RR and control corn grain. Although this study was extensive, a drawback was the low level of corn since it has 8-11% protein.

A special case of risk assessment was the one carried out on the YieldGard® Rootworm Corn (MON 863). [48] This crop was evaluated for assuring its safety using rats fed grain during 90-day. Two levels of inclusion (11 % and 33 % w/w) of MON 863 were tested and compared with conventional corn varieties. Body weight, food consumption, clinical pathology parameters, organ weights gross and microscopic appearance of tissues were compared between diets. Several differences appeared on animals fed GM and Non-GM diets. To 33 % of inclusion level a slight increase in male white blood cell count, lymphocyte and absolute basophiles was found. Also a slight increase in glucose (MON 963 females) and decrease in chloride (MON 863 males) was observed. A minor incidence of kidney tubule mineralization (MON 863 females) and high incidences of focal inflammation and tubular regenerative changes in the kidneys (MON 863 males) was also found. Despite these differences, the authors argued that most of the microscopic findings were of minimal severity or the values were within the range of the individual animal reference control values.

At the previous described MON 863 study, none of the pathology findings were considered to be attributable to the tested crop. After that, data were released and re-analyzed. [49] When appropriate statistics were added, then slight but dose-related significant variations in growth were observed. Signs of hepatorenal toxicity were revealed by chemistry measurements; triglycerides increased in females and urine phosphorous and sodium excretions diminished

in males. Therefore, the two main organs of detoxification, liver and kidney, were disturbed. The results suggest the need of new analysis before concluding that MON863 is safe as food for animals or humans.

Study ***Case 3: GM rice***

The rice variety expressing the kidney bean *Phaseolus vulgaris* lectin agglutinin E-form (PHA-E lectin) was evaluated through 90-day animal study. [50] Female Wistar rats were given a nutritionally balanced (regarding micro- and macronutrients) purified diet with 60% parental rice, 60% PHA-E rice or 60% PHA-E rice spiked with 0.1% recombinant PHA-E lectin. A preceding 28-day *in vivo* study was included to determine the toxicity of the pure PHA-E lectin. Biological, biochemical, microbiological and pathological parameters were examined. Food consumption was measured throughout the study and showed a greater absolute and relative consumption of PHA-E rice. These differences can affect growth and physiological parameters and could cause secondary adverse effects and thereby mask adverse effects caused by the test product itself. Also, absolute and relative weight of the small intestine, stomach and pancreas were significantly higher in the rats fed with PHA-E rice and PHA-E rice spiked with PHA-E lectin. Since significant differences were observed in both groups fed PHA-E rice and PHA-E rice spiked with PHA-E lectin compared to the control rice group, the observed effects could be caused either by the PHA-E lectin. However, since a dose-response relationship was not seen for some parameters, there is a possibility that the observed effects were caused by some kind of secondary effect of the genetic modification, not picked up by the compositional analysis.

Similar experimental design to that of Poulsen [50] was applied to assess the safety of a rice variety expressing the snowdrop *Galanthus nivales* (GNA lectin), [51] except the preceding 28-day *in vivo* study to determine the toxicity of the pure lectin. Ranges of clinical, biological, immunological, microbiological and pathological parameters were examined. There was no statistically significant

difference in food consumption between groups. However, a marked significantly higher relative water intake was seen in both males and females given GNA rice. The authors attribute some hematological differences to this increased water intake. A statistically significant increase in the relative weight of the small intestine (+10%) was observed in female rats fed on GNA rice, as well as an increase in absolute and relative weight of the adrenals. Significantly higher level of alanine aminotranferase was observed in females fed GNA rice and could indicate some kind of effect on the liver.

Schroder et al., [52] tested the transgenic KMD1 rice expressing Cry1Ab protein, compared to its non-transgenic parental wild type. No differences on weight gain were observed during the study. At the histopathological examination, minor changes were reported and not attributable to KMD1 rice. Similar to the study of RR soybean, testing the potential toxic effects using mouse testes as a sensitive biomonitor, [35] Bt-corn was analyzed. [53] The authors conclude that ingestion of Bt corn in a nutritionally balanced diet by the mother during pregnancy and lactation and later by the young developing male mouse had no negative effect on fetal, postnatal, pubertal, or adult testicular development or body growth.

Other study cases

Chen et al., [11] assessed the safety of GM sweet pepper and tomato expressing the cucumber mosaic virus (CMV) coat protein (CP) gene. The following tests were conducted: acute toxicity assay (LD50), micronucleus test, sperm aberration test, Ames test and bioassay of 30-day feeding period. It was found that these two GM products showed no genotoxicity either *in vitro* or *in vivo* by the micronucleus test, sperm aberration test and Ames test. Animal feeding trials of 30 d in 3 week old rats showed no significant differences in growth, body weight gain, food consumption, hematology, blood biochemical indices, organ weights and histopathology either on GM sweet pepper or tomato diets compared to those with non-GM diets. The authors concluded that the

CMV-resistant sweet pepper and tomato were comparable to the non-GM counterparts in terms of food safety.

Currently, there are other GM crops that have been evaluated for safety. Examples are the Canola GT200 and GT73 tested in rainbow trouts [54] and peas tested in mice. [55] None of these studies has found differences attributable to genetic modification.

A view of findings

As time progresses, studies on risk assessment of GM foods have improved. According to changes in the guidelines, the publications are also moving towards sensible and specific indicators about the security of GM foods intake. In publications reviewed by Pryme and Lembcke, [28] and the previously mentioned in this review, the conclusions have varied from not alteration of nutritional value of GM food tested, [11, 32, 35, 44, 45, 47, 53, 56] minimal detrimental effects on the nutritional value, [57] to some *in vivo* effects in mice and rats, [20, 37, 38, 58]

Animal models to test GM foods have been diverse, including rats, mice, cattle, fish and poultry, and the assay periods also varied. Some of the researches carried out [44-46] are not true risk assessment because they do not study the effects in the animals through microscopic, biochemical or *in vivo* indicators. These studies only analyze the performance of animals respect to body weight, food ingested or milk production; therefore, they are more agronomic than risk assessment studies. Even though the conclusions of these studies are true, because the GM food did not influence the performance of animals, they could be misleading. The common result is that there are no effects at the macroscopic level. Some others [35, 53] analyzed the effects at inheritance level or the testicular development, but not in organs or specific tissues previously reported with alterations due to GM foods.

Even in the studies that found adverse effects, there were no observable macroscopic changes on body or organs weight. However, organelles and other

sub cellular structures were clearly affected, as it was shown at ultramicroscopic levels. [36-41, 43, 48-51] Previously, it was established the necessity to test GM crops case by case. The efforts should be directed to find the best experimental design considering the inclusion levels of GM food that with the animal model effects can be detected as they could happen in the human organism. Just one model seems to be insufficient to assure health safety due to the metabolic differences of each species, which could mask or hide adverse effects. New methodology proposed by Poulsen et al., [50] results in an interesting strategy which is more sensitive and specific, compared to the one currently used for GM crops, to optimize the sensitivity and specificity of a bioassay.

Another important point is the level of inclusion of the tested food. It is well known that the evaluation of a whole food has complications for the diet formulation. Some imbalance could occur by the inherent components of the GM food. Almost all of the studies mentioned in this review fulfill this condition, nevertheless; the case of soybean deserves special attention. Moreover, derived and processed products have arrived at the food chain, especially for soybean. This grain is industrially processed and used in several presentations, as the protein concentrates and isolates. Then although the evaluation of the whole grain is suitable, also the evaluation of its different industrial products must be considered. Additionally, the difficulty to acquire the non-GM parent line of the GM-crop tested is another problem. Sometimes it is not possible to attribute the cause of the difference observed between the Non-GM and GM groups to the GM food or to the differing nutrient and anti-nutrient compositions of each crop caused by genetic and/or environmental influences.

The new recommendations suggest that *in vitro* methods can serve either as screening systems to assess potential toxicity of a compound, or for investigations of a toxicological mechanism underlying a specific effect observed *in vivo* or predicted from the structure of a molecule. That is to say, the observed adverse effects in the studies must thoroughly be investigated. It is probable that the nutritional status indicators are not sufficiently sensitive to detect changes in the organism. The above mentioned findings and their implications call for a

retaking and rethinking of the process of risk assessment. For example, the substantial equivalence principle, in the future will not be applicable for some GM foods, since these are being modified in their nutrimental composition to fight specific diseases. It is not matter to discard the previous studies, but to exhaust the possibilities to prove the security of transgenic foods. The offered advantages by transgenic foods are important for many problems but it is necessary to prove that those are not bringing about another.

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CAPÍTULO 2

Caerulein-induced pancreatitis in rats: Histological and genetic expression changes from acute phase to recuperation

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BASIC RESEARCH

Caerulin-induced pancreatitis in rats: Histological and genetic expression changes from acute phase to recuperation

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Abstract

AIM: To study the histological and pancreatitis-associated protein mRNA accumulation changes of pancreas from acute phase of caerulin-induced pancreatitis to recuperation in rats.

METHODS: Acute pancreatitis was induced by caerulein in male Wistar rats and followed up for 90 d by histological and mRNA analyses of pancreas. Pancreases were dissected at 0, 9, 24 h and 3, 5, 15, 30, 60, 90 d post-induction. Edema (E), polymorphonuclear neutrophil (PMN) infiltration, cytoplasmic vacuolization (V), zymogen granule depletion (ZD) and acinar disorganization (AD) were microscopically evaluated. Accumulation of pancreatitis-associated protein (PAP) and L13A mRNAs were quantified by real-time PCR.

RESULTS: The main histological changes appeared at 9 h post-induction for PMN infiltration and cytoplasmic V, while at 24 h and 3 d for E and ZD, respectively. All the parameters were recovered after 5 d, except for ZD which delayed more than 30 d. The main AD was observed after 15 d and values returned to normal after 30 d. Similarly to histological changes, accumulation of the PAP mRNA was increased at 9 h with the highest accumulation at 24 h and differences disappeared after 5 d.

CONCLUSION: From the acute phase to recuperation of pancreatitis, regeneration and re-differentiation of pancreas occur and PAP expression is exclusively an acute response of pancreatitis.

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Key words: Acute pancreatitis; Histological changes; PAP mRNA; L13A mRNA

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INTRODUCTION

Acute pancreatitis (AP) is an inflammatory disease of the pancreas associated with autodigestion of the gland, as a consequence of the intrapancreatic activation and releasing of digestive enzymes[1]. The pathogenic mechanisms involved in the development of acute pancreatitis are not well understood[2,3] although many studies on it are available. The acute period of pancreatitis is characterized by exocrine insufficiency as a result of important morphological alterations and changes on the expression of a number of genes in the pancreas[4,5]. If pancreatitis is not fatal, a stepwise regeneration of the morphology is followed[6].

Some events that regulate the severity of acute pancreatitis are known, involving common inflammatory and repair pathways[7,8]. Recently it was demonstrated that pancreatitis-associated protein (PAP), an acute phase protein, is itself an important determinant of disease severity[9]. The expression of this gene is low in the normal pancreas and becomes strongly augmentation after even mild pancreatic inflammation[10]. This augmentation is assumed in AP induced by caerulein, although it can be assayed in AP induced by retrograde injection of sodium taurocholate. Currently, pancreatitis is induced by supramaximal dose of caerulein and there is no report yet on histological and PAP mRNA changes in the pancreas as response of AP induction.

Therefore, the objective of this study was to evaluate the morphological and PAP mRNA changes in the acute and adaptive phases of pancreatitis induced by caerulein in rats. Morphological changes were evaluated by light microscopy and PAP mRNA levels by real-time PCR.

MATERIALS AND METHODS

Bioassay

The experiments were carried out on 33 male Wistar rats (mean weight 100.8 g and 4-wk old). The animals were fed with standard laboratory chow and water *ad libitum*

under controlled room conditions. Acute pancreatitis was induced according to the method of Dusseti *et al*[11]. The rats were injected intraperitoneally with caerulein (Sigma Chemical Co., St. Louis, MO, USA) at a dose of 50 μg/kg of body weight per hour for 6 h. The rats were sacrificed at h 9 and 24, and on d 3, 5, 15, 30, 60 and 90 respectively. Three control rats without caerulein injection were sacrificed at h 0 and on d 30 and 90 and dissected. Prior to sacrifice, the rats were anesthetized with 10-15 mg of tiletamine chlorohydrate and zolazepam chlorohydrate (Zoletil 50®) per kg of body weight. Pancreas was dissected for histological or RNA analysis. Feeding protocol and animal handling were approved by the local ethics committee.

Histological examination

Pancreatic sections were fixed in 10% formaldehyde, embedded in paraffin, cut (3-5 μm in thickness) with a semi-motorized rotary microtome (Leica RM-2145) and mounted onto slides. The sections were processed for H&E staining and examined by conventional light microscopy (Olympus microscope B × 50) with 20 ×, 40 × and 100 × objectives. Eight random images by objective were recorded by microscope video camera. A pathologist who was blinded to the treatment protocol scored the tissues according to Kyogoku *et al*[12] for zymogen granule depletion (0, absent; 1, less than 20%; 2, 20%-50%; 3, more than 50%), interstitial edema (0, absent; 1, expanded interlobular septa; 2, expanded intralobular septa; 3, separated individual acini), polymorphonuclear neutrophil (PMN) infiltration (0, absent; 1, less than 20 PMNs per intermediate power field (IPF); 2, 20-50 PMNs per IPF and 3, more than 50 PMNs per IPF), grade of vacuolization based on the percentage of acinar cells with cytoplasmic vacuoles per IPF (0, absent; 1, less than 20%; 2, 20%-50%; 3, more than 50%) and acinar disorganization based on the percentage of area without normal acinar distribution (0, absent; 1, less than 20%; 2, 20%-50%; 3, more than 50%).

RNA preparation

Extracted pancreas was immediately rinsed with RNase-free water (treated with 0.05% diethyl pyrocarbonate, DEPC), homogenized in 1 mL of TRIzol® reagent (GIBCO-BRL, Grand Island, NY, USA), frozen and stored at -70℃. Total RNA was extracted by phenol/chloroform extraction and isopropanol precipitation[13]. Accumulation of PAP mRNA (GenBank access No. NM_053289.1) was analyzed as indicator of pancreatic injury. L13A mRNA (GenBank access No. X68282) was also analyzed as housekeeping gene presumed to be invariant[14]. All the primers were designed with Primers3 software and synthesized by Sigma Genosys (Woodlands, TX, USA). Their sequences and PCR product sizes are listed in Table 1. Prior to the reverse transcription reaction, potentially contaminating residual genomic DNA was eliminated with DNase I (Invitrogen, Carlsbad, CA, USA) followed by reverse-transcribed using specific primers and SYBR Green RT-PCR reagents (PE Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Synthesized complementary DNA (cDNA) corresponding to 100 ng total RNA was used for real-time PCR.

[illegible]

Primer	Sequence	GenBank No.	Product size (bp)
PAPfw	5' TGAATTATGTCAACTGGGAGAGG 3'	NM-053289.1	318
PAPrv	5' TTACTGCTTTGCAAGACATGAGG 3'		
rL13A/Fw	5' AAGCAGGTACTGCTGGG 3'	X68282	261
rL13A/Rv	5' CCAACACCTTGAGGCGTT 3'		

Standard synthesis

Standards for the absolute quantification for PAP and L13A mRNAs were synthesized by Titan one Tube RT-PCR system (Roche Biochemicals, Indianapolis, IN, USA) with the same PCR parameters described above, according to the manufacturer's instructions. Then, PCR products were purified with Wizard® SV gel and PCR clean-up system (Promega, Madison, WI, USA) and quantified by fluorometry.

Quantitative PCR analysis

Real-time quantification of PAP and L13A mRNAs was performed with an iCycler iQ detection system (BIO-RAD, Hercules, CA, USA). SYBR Green I assay was used for quantification of all specific genes. For PAP mRNA quantification of each 50 μL SYBR Green PCR, 9.0 μL cDNA (corresponding to 100 ng total RNA), 1.8 μL sense primer (10 μmol/L), 1.8 μL antisense primer (10 μmol/L), 25 μL SYBR Green PCR Master Mix (PE Applied Biosystems) and 12.4 μL PCR-grade water were mixed together. The PCR conditions were 1 cycle at 94℃ for 4 min, then 35 cycles at 94℃ for 30 s, at 61℃ for 30 s and at 70℃ for 30 s, with a single fluorescence measurement at the end of the 70℃ for 30 s. For L13A mRNA quantification of each 50 μL SYBR Green PCR, 9.0 μL cDNA (corresponding to 100 ng total RNA), 1.2 μL sense primer (10 μmol/L), 1.2 μL anti-sense primer (10 μmol/L), 25 μL SYBR Green PCR Master Mix (PE Applied Biosystems) and 13.6 μL PCR-grade water were mixed together. The PCR conditions were 1 cycle at 94℃ for 3 min, then 38 cycles at 94℃ for 45 s, at 55℃ for 45 s and at 72℃ for 1 min, with a single fluorescence measurement at the end of the 72℃ for 30 s. In both cases, a melting curve program (60-95℃ with a heating rate of 0.5℃ for 30 s and continuous fluorescence measurement) and a cooling step to 4℃ were added.

Analysis and normalization of real-time PCR data

Optical data obtained by real-time PCR were analyzed by using the default and variable parameters available in the software provided with the iCycler iQ optical system (BIO-RAD, Hercules, CA, USA). The PCR threshold cycle number (CT) for each cDNA standard and cDNA sample was calculated at the point where the fluorescence exceeded the threshold limit. The threshold limit was fixed along the linear logarithmic phase of the fluorescence curves at 10-20 SDs above the average background fluorescence. The number of amplicon cDNA copies was expressed relative to the amount of total cDNA present (ng). Although it might be difficult to determine the absolute amount of a

Table 2 Histological changes in the pancreas of rats with caerulein-induced acute pancreatitis (mean ± SE)

Period	Zymogen granule depletion	Edema	PMN infiltration	Vacuolization	Acinar disorganization
0 h	0.25 ± 0.23^{a,b}	1.25 ± 0.31^{a,b}	0.00 ± 0.14^{a}	0.25 ± 0.26^{a}	0.5 ± 0.36^{a}
9 h	1.00 ± 0.23^{b,c}	2.25 ± 0.31^{b,c}	0.75 ± 0.14^{b}	2.75 ± 0.26^{c}	0.75 ± 0.36^{a,b}
24 h	1.40 ± 0.20^{c}	3.00 ± 0.28^{c}	0.60 ± 0.12^{b}	2.00 ± 0.24^{b}	1.00 ± 0.32^{a,b}
3 d	3.00 ± 0.18^{e}	2.17 ± 0.25^{b,c}	0.67 ± 0.11^{b}	2.00 ± 0.22^{b}	1.33 ± 0.29^{a,b}
5 d	2.00 ± 0.23^{c,d}	1.25 ± 0.31^{a,b}	0.00 ± 0.14^{a}	1.00 ± 0.26^{a,b}	0.75 ± 0.36^{a,b}
15 d	2.57 ± 0.17^{d,e}	0.86 ± 0.23^{a}	0.00 ± 0.10^{a}	0.00 ± 0.20^{a}	2.14 ± 0.28^{b}
30 d	1.60 ± 0.20^{c}	0.60 ± 0.83^{a}	0.00 ± 0.12^{a}	0.00 ± 0.24^{a}	0.80 ± 0.33^{a,b}
60 d	0.00 ± 0.19^{a}	0.83 ± 0.25^{a}	0.00 ± 0.11^{a}	0.17 ± 0.22^{a}	0.66 ± 0.29^{a}
90 d	0.25 ± 0.13^{a,b}	0.42 ± 0.18^{a}	0.00 ± 0.07^{a}	0.00 ± 0.15^{a}	0.92 ± 0.31^{a,b}

Different letters within a same column differ significantly (P < 0.05)

cDNA present in different samples, quantification of test mRNA transcripts was normalized to a reference gene, the L13A gene.

Statistic analysis

Histological data were expressed as mean ± SE of the scores assigned by the pathologist and evaluated by ANOVA. Also, relation between PAP and L13A after absolute quantification was expressed as mean ± SE and compared using ANOVA test. P < 0.05 was considered statistically significant. NCSS 2001 statistical program was used.

RESULTS

Histological examination

At the beginning (0 h), the architecture of pancreas was normal (Figure 1) with acinar cells exhibiting the typical epithelial polarity. Basal portion of the cells contained their nuclei and apical portion of the secretory vesicles and zymogen granules. Acinar cells in AP displayed moderate vacuolization and light inflammatory infiltration of neutrophils, with the highest score after 9 h post-induction. After 5 d, levels returned to the normal scores maintained to the end of the study. Edema was increased 9 h post-induction with the highest score at 24 h and returned to the normal level on d 5. Zymogen granule depletion was most pronounced on d 3 with later recovery. However, an additional depletion occurred on d 15, returned to the normal levels 30 d post-induction (Table 2). Although some degree of acinar disorganization was observed in the pancreas 9 h after induction, a significant increase was observed on d 15, and returned to the normal level 30 d post-induction (Table 2).

Specific amplification

Melting curve analysis demonstrated that each of the primer pairs (Table 1) amplified a single predominant product with a distinct melting temperature (Tm) as shown for L13A and PAP cDNA in Figure 2. The Tm of products could be seen clearly as a peak in a first derivative plot. The rapid fall of Tm at 87.5℃ and 82.0℃ for L13A and PAP cDNA respectively indicated the presence of a specific product melting at this temperature.

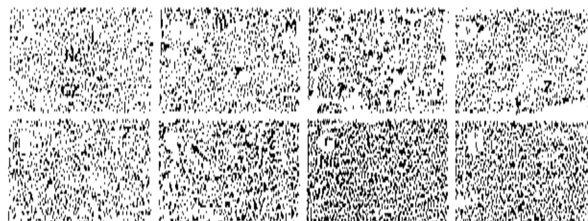

Figure 1 Light-microscopic sections of pancreas (X 133) from 0 h (**A**) and rats with caerulein-induced acute pancreatitis at 9 h (**B**), 24 h (**C**), 3 d (**D**), 5 d (**E**), 15 d (**F**), 30 d (**G**) and 60 d (**H**). Normal pancreatic acinus (0 h) shows zymogen granules (GZ) concentrated in the apical pole of the cell close to the acinar lumen (L). The basal region of the cell contains nucleus (N). In AP, PMN infiltration (M), vacuoles (arrowhead), edema (star), zymogen granule depletion (z) and acinar disorganization (arrow) were observed on d 15. On d 30, pancreatic acinus appearance was almost indistinguishable from that at 0 h.

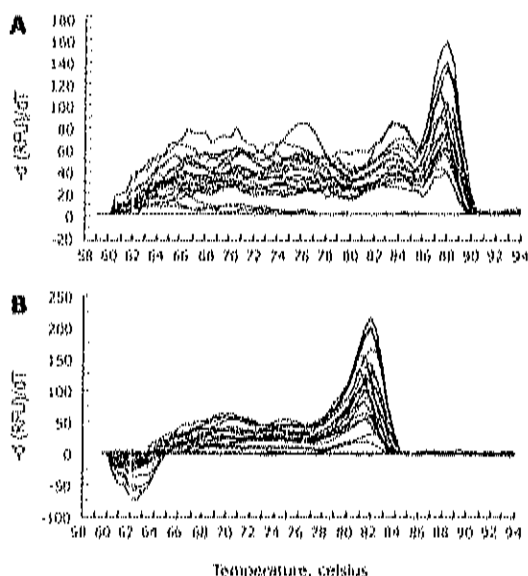

Figure 2 DNA melting curve analysis of amplification products for PAP mRNA (**A**) and L13A (**B**).

Absolute detection and quantification

The absolute quantification was estimated from real-time PCR analyses using a standard curve of tenfold serial dilutions ranging from 1.78 × 10^9 copies to 1.78 × 10^5 copies of L13A mRNA and 1.46 × 10^9 copies to 1.46 ×10^5 copies of PAP mRNA, in 50 μL of volume reaction. As shown in Figure 3, the mRNA of PAP was detected in basal levels without caerulein (0 h). However, after induction of AP, the PAP mRNA expression was slightly augmented after 9 h, and 3.8-fold the basal level at 24 h. After 5 d, differences disappeared and the accumulation of PAP mRNA was decreased gradually to the basal level.

DISCUSSION

The acute phase of pancreatitis is characterized by a pattern of changes in the morphology of exocrine pancreas, expression of secretory proteins and mRNA levels of different genes[1,6,15,16]. The observed changes in our study resemble a mild form of AP, characterized by edematous fluid in the extra-cellular space, causing separation of lobules

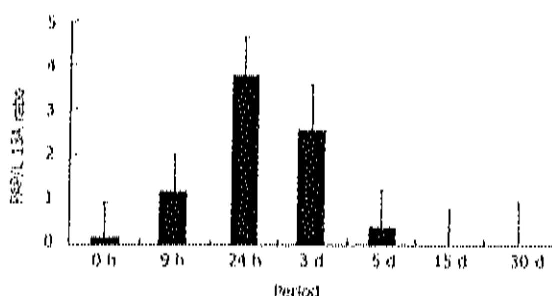

Figure 3 Relative quantification of PAP mRNA in caerulein-induced pancreatitis. Absolute quantification of PAP mRNA as copies was normalized to copies of L13A as determined from standard curves. All data are mean ± SE.

and acini[6]. Usually there is mild invasion by neutrophils, the acinar cells lose their zymogen granules, enlarging the acinar lumen. However, few studies[17-19] have analyzed the morphological changes after the acute phase of pancreatic response.

After acute response, re-differentiation of acinar cells and transition begin with regular acinar cell replete with dense zymogen granules beside cells with a smaller quantity of zymogen granules, on the same microscopic preparation. Intermediate cells change the typical architecture of the pancreas due to the loss of zymogen granules in a particular section. Additionally, the rough endoplasmic reticulum might be reduced and/or rearranged[6]. In this study, zymogen granule depletion and increase of acinar disorganization were presented after the acute phase of pancreatitis (determined by maximal PAP synthesis and its mRNA accumulation according to Iovanna *et al*[4] and our results, respectively). These changes are explained in the context of reconstitution of the normal morphology and function of the pancreas.

De Lisle and Logsdon[20] have shown the differentiation and re-differentiation of mouse pancreatic acinar cells. They immunolabeled acinar cells with monoclonal antibodies specific for acinar or duct cells and showed that more than 97% of the labeled area is acinar positive on d 3, which decreases to approximately 16% on d 9, and then returns to over 97% by day 21 of culture[20]. Lechene de la Porte *et al*[21] induced pancreatitis by the injection of trypsin in multiple sites of the rat pancreas and reported that pancreatic repair involves proliferation of cells from intact acini and tubular complexes. This process of differentiation and re-differentiation of pancreatic acinar cells explains our finding of the highest score for acinar disorganization on d 3 and 15 as well as complete recovering of zymogen granules 30 d post-induction.

In pancreatitis not only morphological changes were observed, but also molecular modifications occurred. Iovanna *et al*[4] showed that PAP is significantly increased 48 h after induction of pancreatitis by retrograde injection of sodium taurocholate in rats, compared to normal pancreas. In spite of methodological differences in the quantified PAP, our results agree with those of Iovanna *et al*[4]. However, PAP mRNA accumulation is quite different between both studies. Differences could be attributable to the pancreatitis induction method, the severity of the problem, as well as the quantification method itself. In our study, after absolute quantification, values were normalized in respect to a housekeeping gene (L13A). Therefore, quantification by real-time PCR may better reflect the actual physiological changes.

Histology of pancreas showed a mild edematous pancreatitis induced by caerulein and the acute response was followed by recuperation process after 15 d of induction, according to acinar disorganization and zymogen granule depletion scores. Although PAP is related to the severity of pancreatitis, its function *in vivo* is not well established. Our findings support that PAP is a protein of acute response, because changes in levels are not observed in reconstitution of the pancreatic normal morphology. In addition, the PAP mRNA changes in AP induced by caerulein, measured by a most sensible technique of quantification as real-time PCR, are similar to those from AP induced by sodium taurocholate.

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CAPÍTULO 3

Pancreatic response of rats fed with genetically modified soybean

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Pancreatic response of rats fed with genetically modified soybean

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Running title: Pancreas response to genetically modified soy.

Key words: genetically modified soybean, pancreas, histological changes, trypsinogens and PAP mRNA, real-time PCR

ABSTRACT

Mice fed genetically modified (GM) soybean were not affected in nutritional performance, but pancreatic microscopic features were disturbed. The mechanisms for these contradictory findings are unknown. This study was designed to investigate the changes of histological characteristics of acinar pancreatic cells and their expression of pancreatitis-associated protein (PAP) and trypsinogens mRNA in rats fed GM soy protein. Two bioassays were run, each one with 34 Wistar rats distributed into 2 groups fed with Non-GM or GM-soy protein (18 % protein) for 0, 1, 3, 5, 15 and 30 days. Nutritional evaluation, pancreatic histological analysis and quantification of PAP and trypsinogens mRNAs levels using quantitative RT-PCR by real-time were done. Plasma amylase was also measured. There were no differences in nutritional performance between rats fed Non-GM and GM diets. The GM diet induced zymogens-granules depletion after 15 days feeding, returning to normal levels after 30 days, compared to the Non-GM diet ($p < 0.05$). Acinar disorganization was detected with the GM diet after five days and it was recovered thereafter. Levels of PAP mRNA significantly increased in the GM diet between the first and third day and decreased to basal level by the fifteen day Tryspsinogens mRNA peak at two different times; at day one and at day fifteen, decreasing to control levels after thirty days. Plasma amylase progressively increased along the assay. This indicates that GM soy protein intake affected pancreas function, responding with early acute PAP mRNA increase and cellular changes followed by recuperation of acinar cells, after 30-days.

INTRODUCTION

Genetically modified (GM) crops are currently exploited around the world both for animal and human consumption. The main biotech crop is soybean, with 58.6 million hectares (57% of global biotech area) in 2006 [James, 2006]. Herbicide tolerance is the dominant GM trait, followed by insect resistance, using different genes for conferring each trait. Despite production benefits, the commercialization of biotech crops has raised concern and protests from consumers, environmental groups, some governments and scientists. Proponents of agricultural biotechnology have suggested that this debate should be resolved by profound scientific knowledge [Frewer et al., 2004].

Current concerns about the effects of GM foods on nutrition and health are largely due to lack of deeper knowledge, since there are few scientific papers on the subject. This contrasts to the more than one thousand published papers about other aspects of GM crops including detection, new modifications, consumer acceptance, regulations, opinions and environmental risks (http://www.ncbi.nlm.nih.gov/pubmed). Additionally, nutritional evaluation studies are quite heterogeneous. GM- corn [Barriere et al., 2001, Brake et al., 2003, Brake et al., 1998, Donkin et al., 2003, Erickson et al., 2003, Hammond et al., 2004, Hammond et al., 2006, Ipharraguerre et al., 2003, Seralini et al., 2007, Yonemochi et al., 2002], potatoes [Hashimoto et al., 1999, Seek et al., 2005], tomato [Chen et al., 2003], soybean [Hammond et al., 1996] and other crops [Melander et al., 2003], have been macroscopically evaluated using different experimental conditions, and no adverse effects have been found. However, at the microscopic and ultramicroscopic levels, changes attributable to GM food intake have been reported

[Ewen et al., 1999, Fares et al., 1998, Malatesta et al., 2003, Malatesta et al., 2002, Malatesta et al., 2002, Tudisco et al., 2006, Vecchio et al., 2004].

Mice chronically fed since gestation with a diet containing GM soybean had problems in zymogens synthesis and processing by pancreatic acinar cells [Malatesta et al., 2002b]. Additionally, reduced nucleoplasmic and nucleolar splicing factors and perichromatin granule accumulation on pancreatic acinar cell nuclei were observed [Malatesta et al., 2003]. Moreover, changes found in their hepatocytes suggest high metabolic rate and intense molecular trafficking [Malatesta et al., 2002a]. Lactic dehydrogenase (mainly the LDH1 isoenzyme) significantly increased in kidneys and hearts of rabbits fed a GM soybean-containing diet [Tudisco et al., 2006]. This suggests that GM soybean affects animal physiology, without modifying gross indicators such as total body and fresh organ weights.

As mentioned before, GM soybean feeding induces granules depletion, amylase activity reduction and chromatin changes. These effects also occur in animal models of pancreatitis [Andrzejewska et al., 2005, Ding et al., 2003, Magaña-Gómez et al., 2006]. In addition, pancreatitis-associated protein (PAP), a secretory protein not normally expressed in pancreas is highly induced by acute pancreatitis [Dusetti et al., 1995, Iovanna et al., 1991, Magaña-Gómez et al., 2006]. PAP mediates protection against pancreatic injury by suppression of the local pancreatic and systemic inflammation during acute pancreatitis [Zhang et al., 2004]. High PAP levels in serum or in pancreatic juice may indicate a pancreatic response to cellular injury [Paajanen et al., 1999]. Additionally, alterations of gene expression of other proteins like trypsinogens occur during pancreatitis [Iovanna et al., 1991]. Trypsinogens are an important constituent of zymogen granules and source of trypsin for protein digestion at the small intestine.

Based on the above mentioned information, we propose that GM soybean intake induces pancreatic stress or injury. This study was designed to investigate cellular structure of acinar pancreatic cells and the expression of pancreatitis-associated protein (PAP) and trypsinogens by quantification using qRT-PCR by real-time PCR in rats fed GM soy protein for 30 days.

MATERIALS AND METHODS

Materials. Two commercial soy protein isolates (SPI) labeled as GM (SUPRO 500E) or Non-GM (SUPRO 500E IP) were purchased and GM or non-GM origins of the samples were confirmed. Certified reference material from the Institute for Reference Materials and Measurements, of soybean powder containing 5% Roundup Ready™ soybean (Fluka Chemie, Geel, Belgium) was used as positive control for DNA characterization. Genomic DNA was isolated as previously described by Magaña-Gómez et al. [2003]. Four different DNA fragments were detected by PCR; soybean lectin as an endogenous reference control and specific sequences from the transgenic construct: CaMV promoter 35S (p35S), 5-enol-pyruvylshikimate-3-phospate synthase (CP4-EPSPS) that confer resistance to the herbicide glyphosate, as event-specific transgene and the NOS terminator (NOS-T) derived from *Agrobacterium tumefaciens*. All PCR reactions contained 1 U recombinant Taq DNA Polymerase (Invitrogen corp. Carlsbad, CA, USA) in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.8 mM $MgCl_2$, 200 μM each dNTP, and 0.32 μM of forward and reverse primers in 25 μL volume. Genomic DNA (200 ng) was used as template. PCR conditions were 40 cycles of 30 s at 94 °C, 35 s at 63 °C, and 40 s at 72 °C, with 3 min at 94 °C prior to the reaction and 5 min of final extension at 72 °C in a MJ Mini Thermal Cycler (BIO-RAD, Hercules, CA, USA).

PCR products were resolved in a 1% agarose gel prestained with ethidium bromide (0.1 μg/mL). Nucleotide sequences of the primers used are listed in Table 2. Additionally, in both soy protein isolates, physiologically-active components as trypsin and chymotrypsin inhibitors [Oppert et al., 1997] and isoflavones genistein and daidzein [Klump et al., 2001] were measured. SDS-PAGE of the both soy protein isolates were also analyzed [Laemmli, 1970].

Experimental design. The experimental design consisted of diets made from GM- or Non-GM-soy protein isolate (SPI) to feed male rats during 30 days. Nutritional quality, trypsin and chymotripsin inhibitors' activity, isoflavones content and recombinant DNA presence were determined in the SPIs. Rats were fed GM or Non-GM diet and pancreatic histology, gene expression and amylase levels were analyzed along the bioassay. Cellular damage was detected by histology and mRNA levels of pancreatitis-associated protein (PAP) and trypsinogen were measured as indicator of pancreatic injury.

Bioassay. Two independent bioassays of 34 male Wistar rats each, mean weight 47.8 g and four weeks old (bioassay 1) or 62.6 g and five weeks old (bioassay 2), were done. After random distribution into two groups of 17 rats each, the animals were individually housed in metabolic cages (NALGENE). Each group was fed an isoenergetic diet of conventional (Non-GM) or GM-soy protein isolate (18 % protein) for 0, 1, 3, 5, 15 and 30 days. Composition of diets is shown in Table 1. The bioassay was carried out at 22-25 °C and a 12-h light:dark cycle (lights were on at 07:00 h). Diet and water were given *ad libitum*. Previous to sacrifice, all animals were starved for 9 h and thereafter anesthetized with 10 to 15 mg of tiletamine chlorohydrate and zolazepam chlorohydrate

(Zoletil 50®) per kg of body weight. Blood samples were collected into polypropylene tubes containing EDTA, and plasmas were separated by centrifugation for 20 min at 4 °C, and then stored frozen at – 70 °C until analyzed. Pancreas were dissected and divided in two portions, for histological and gene expression analyses. The results of gene expression analysis were corroborated thorough a second bioassay in similar conditions to the first bioassay. Feeding protocol and animal handling were approved by the local ethics committee.

Diet performance. Nutritional quality of the diets was evaluated in both bioassays by ingested food, feed conversion (FC) and protein efficiency (PE). FC was determined by the weight gained (g)/food intake (g) ratio. PE was obtained by the weight gain (g) divided by protein intake. Both indicators (FC and PE) were calculated from the estimated values of each dietary sub-group at day 30 of the experimental period. Food consumption registries were taken every three days.

Plasma amylase levels. Plasma amylase was determined using a commercial kit from Randox Laboratories LTD (Crumlin, UK) according to the company specifications.

Histological examination. Pancreas sections were fixed in 10% formaldehyde, embedded in paraffin, and sections of 3–5 μm thick were cut with a semi-motorized rotary microtome (Leica RM-2145) and mounted on slides. Sections were processed for H&E staining and examined by conventional light microscopy (Olympus microscope BX50) with 20X and 40X objectives, as previously described in Magaña-Gómez et al. [2006]. Eight random images by objective and feeding period were recorded by microscope video camera. A pathologist, blind to the treatment protocol, scored the tissues according to the methodology by Kyogoku et al. [1992] for zymogens granules

depletion (0, absent; 1, less than 20%; 2, 20-50%; 3, more than 50%); interstitial edema (0, absent; 1, expanded interlobular septa; 2, expanded intralobular septa; 3, separated individual acini); polymorphonuclear neutrophiles (PMN) infiltration (PMNi) (0, absent; 1, less than 20 PMNs per intermediate power field (IPF); 2, 20-50 PMNs per IPF and 3, more than 50 PMNs per IPF), vacuolization, based on the percentage of acinar cells with cytoplasmic vacuoles per IPF (0, absent; 1, less than 20%; 2, 20-50%; 3, more than 50%) and acinar disorganization, based on the percentage of area without normal acinar distribution (0, absent; 1, less than 20%; 2, 20-50%; 3, more than 50%).

Statistical analysis. The results of protease inhibitors and isoflavones, diet performance, plasma amylase and histological examination were expressed as means ± standard error of the mean (SEM). Statistical analysis of the mean values was done using the General Linear Models procedure, followed by comparisons using the Tukey-Kramer multiple comparisons procedure. $P < 0.05$ was considered as significance limit. NCSS 2001 statistical program was used [NCSS, 2001].

RNA preparation and reverse transcription. Dissected pancreas were immediately rinsed with RNase-free water (treated with 0.05% diethyl pyrocarbonate, DEPC), homogenized in 1 mL of TRIzol® Reagent (Invitrogen, Carlsbad, CA, USA), frozen and stored at -70°C. Total RNA was extracted with phenol/chloroform followed by isopropanol precipitation [Chomczynski, 1993]. Prior to reverse transcription (RT), potentially contaminating residual genomic DNA was eliminated with DNase I (Invitrogen, Carlsbad, CA, USA). The RT reaction was done in a total volume of 21 µL containing 2 µg of total RNA, 1 µl of 0.5 mg/mL oligo(dT)15 primer, 40 U of RNaseOUT, 2.5 mM dNTP, 200 U of Moloney murine leukemia virus Reverse Transcriptase (M-MLV RT) and manufacturer's 5X RT Buffer. All components were purchased from Invitrogen

(Carlsbad, CA, USA). Synthesized complementary DNA (cDNA) was stored at −20 °C until use.

Quantitative PCR analysis. Real-time RT-PCR quantification (qRT-PCR) of PAP-, trypsinogen- and L13A mRNAs and 18S rRNA was performed with an iCycler iQ detection system (BIO-RAD, Hercules, CA, USA). Accumulation of PAP mRNA (GenBank accession No. NM_053289.1) was used as an indicator of pancreatic injury as reported elsewhere [Magaña-Gómez et al., 2006]. Trypsinogen mRNA (GenBank accession No. V01274 and V01273) was measured to evaluate exocrine pancreas function. L13A mRNA (GenBank accession No. X68282) and 18S ribosomal RNA (rRNA) (GenBank accession No. X01117) were also analyzed to normalize data in bioassay 1 and 2 respectively [Jesnowski et al., 2002]. All the primers were designed with Primers3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized by Sigma Genosys (The Woodlands, TX, USA); primer sequences and PCR product sizes are listed in Table 2.

SYBR Green I was used for all genes quantification. For 25 µL of reaction, 1 µL of cDNA (corresponding to 100 ng of the original total RNA), 0.9 µL forward primer (10 µM), 0,9 µL reverse primer (10 µM), 12.5 µL SYBR Green PCR Master Mix (PE Applied Biosystems) and 9.7 µL PCR-grade water, were mixed. The PCR parameters for quantifying PAP mRNA levels were 94 °C for 4 min, 1 cycle, then 94 °C for 30 s, 61 °C for 30 s and 70 °C for 30 s, 40 cycles. Parameters for trypsinogens mRNA quantification were 94 °C for 6 min, 1 cycle, then 94 °C for 35 s, 58 °C for 35 s and 72 °C for 40 s, 40 cycles. The PCR parameters for L13A quantification were 94 °C for 4 min, 1 cycle, then 94 °C for 30 s, 68 °C for 35 s and 70 °C for 40 s, 6 cycles, then -0.3 °C from 68 °C for 35

s and 70 °C for 40 s, 34 cycles. The PCR parameters for *18SrRNA* quantification were 85 °C for 30 s, 94 °C for 6 min, 1 cycle, then 94 °C for 35 s, 60 °C for 35 s and 72 °C for 40 s, 40 cycles. In all cases, a single fluorescence measurement was made at the end of each extension segment. A melting curve program (60–94.5° C with a heating rate of 0.3 °C, 20 s with continuous fluorescence measurement) and a cooling step to 4 °C, were added.

Relative abundance of mRNA in each pancreatic section was measured according to the Pfaffl method [Pfaffl, 2001], with the next formula.

$$\textit{Ratio of gene expression} = \frac{(E_{target})^{\Delta C_{t,\ target\ (control - sample)}}}{(E_{ref})^{\Delta C_{t,\ ref\ (control - sample)}}}$$

The above algorithm was used to calculate the results from amplification, where E_{target} and E_{ref} are the efficiencies of the target (PAP and Trypsinogens) and reference genes (L13A and 18S) respectively and ΔC_t is the difference between the mean C_t of control cDNA (Non-GM diet) and sample cDNA (GM diet). To confirm our results, we also tried the Absolute Fluorescence Increase method using the LinRegPCR software v.7.5 which measures the actual efficiency of each amplification curve by fitting its linear part in a simulation plot of the Log (fluorescence) versus Cycle in the exponential phase of amplification and calculates the efficiency from the slope of a linear regression model of the simulation curve [Pfaffl, 2001]. All the amplifications had PCR efficiencies from 1.9 to 2.0 and correlation coefficients above 0.999. Ratios of expression values for GM relative to Non-GM diet for each period were plotted in Excel (Microsoft, Redmond WA).

RESULTS

Sample characterization. Specific PCR products were obtained (Figure 1). The soybean lectin gene (157 bp) was detected in all the samples except for the no-template control (NTC). Fragments of 180, 218 and 125 bp (CP4-EPSPS, CaMVp35S and T-NOS, respectively) were obtained with SUPRO 500E and positive control, but not in SUPRO 500E IP and NTC. These data confirmed that SUPRO 500E IP was Non-GM and SUPRO 500E was prepared from glyphosate tolerant soybean (GTS).

Additionally, both SPI samples were analyzed for protein integrity. Their electrophoretic patterns by SDS-PAGE had well defined protein bands without degradation and a very similar pattern in both GM and conventional Non-GM soy isolates (data not shown). Isoflavone levels were different between samples (Table 3), with the highest content in Non-GM SPI for both dadzein and genistein isoflavones. Activity of both trypsin and chymotrypsin inhibitors was similar for GM or Non-GM soybean samples.

Diet performance. Nutritional quality of the diets was similar for Non-GM and GM group in both bioassays. To ensure similar consumption of diets, ingested food was weighed during the bioassays and no differences ($p > 0.05$) were found (Table 4). Body weight gain was similar between groups ($p > 0.05$). Similarly, food conversion and protein efficiency values were comparable ($p > 0.05$) between Non-GM and GM diets during the study and after 30 days feeding (Table 4).

Plasma amylase. Amylase levels were measured in bioassay 1 and the values increased during the bioassay in both diets. However, from day five, lower levels were found in rats from GM diet, (Figure 2).

Histological examination. Normal pancreas architecture was observed with acinar cells at the beginning (0 h) of the experiment (data not shown). The basal portion of the cells contained the nucleus and the apical part, the secretory vesicles and zymogens granules. Pancreas from both rat groups on Non-GM and GM diets did not have edema, infiltration of PMNs nor vacuolization along the bioassay. However, the GM diet induced zymogens granules depletion starting at day five and until the fifteen day being significantly higher compared to the Non-GM diet ($p < 0.05$). However, zymogens granules content was fully recovered by day 30th (Figure 3). Similarly, acinar disorganization was suddenly increased in pancreas of rats fed GM diet on day 5th, remained high until day 15th and was recovered thereafter (Figure 3). Micro-photography of the characteristic histological changes of pancreatic acinar cells is shown in Figure 4.

Quantification of PAP and trypsinogens mRNA. Quantitative RT-PCR analysis was used to evaluate the relative steady-state levels of PAP and trypsinogens mRNA in pancreas of Non-GM or GM fed rats. In both assays, PAP transcripts were detectable since the first day in GM group. Maximal accumulation was detected after 3 days or 1 day feeding in bioassay 1 and 2 respectively, respect to Non-GM group (Figure 5 A, B). After 5 days, PAP mRNA abundance was completely attenuated in bioassay 1, while in bioassay 2 the relative values decreased (GM:Non-GM) by day fifteen and remained similar the rest of the experiment.

Changes in relative accumulation of trypsinogens mRNA had a biphasic pattern. In both bioassays, trypsinogen mRNA index increased (GM:Non-GM) was detected in days 1-3, high in bioassay 1 and modest in bioassay 2. Between days 4 and 5, the index was negative in the bioassay 1, while it was still positive, although small, in bioassay 2. After 15 days feeding, trypsinogen mRNA increased several times in GM respect to Non-GM

diet in both bioassays. On day 30, GM/Non-GM index of trypsinogen mRNA had a reduction at 1 in bioassay 1, although the values were above the control level in bioassay 2 (Figure 6 A, B).

DISCUSSION

Most of the currently available GM plants worldwide contain the cauliflower mosaic virus (CaMV) 35S promoter [Ahmed, 2002]; therefore primer pair for this fragment can be used to screen for GM materials but can not identify specific lines of GM soybean. Thus, construct specific primer pairs were useful in the present study to confirm, previously to the bioassays, the identity of commercial soy protein isolate samples and classify the source of SUPRO 500E as glyphosate tolerant soybean (GTS 40-3-2) This study compared two commercial soy isolates, Non-GM and GM with a quite similar protein pattern. Additionally, the process used to obtain the protein isolate was identical by the same company. Both ingredients are used for food formulations and eaten by consumers.

Similarly to results of other studies [Hammond et al., 1996, Malatesta et al., 2002, Tudisco et al., 2006], animal growth and protein efficiency for both Non-GM and GM soybean diets did not show differences; therefore, genetic modification of soybean does not appear to affect the appropriate nutritional utilization of protein compared to Non-GM. Proteases inhibitors could affect pancreas functions; however, values for trypsin and chymotrypsin inhibitors activity were similar between GM and Non-GM diet, both within the normal range for SPI. Another antinutrient in soybean is lectin; however, it is very labile and it is not normally detectable in soy protein isolates [Calderón de la Barca et al., 1991].

The main soy isoflavones, daidzein and genistein, had a lower concentration in GM compared to Non-GM SPI, but both data were in the normal range for soy protein isolates. Previously, no differences were found for isoflavones between GM soybean and its isogenic line [Padgette et al., 1996]; later, in the same GM soybean varieties an overall reduction of isoflavones was reported [Lappé et al., 1999], as we did. Differences could be due to the origin of the samples, genotype, environment, variety, crop year and growth location [Hoeck et al., 2000, USDA, 2002, Wang et al., 1994]. In safety studies, dietary daidzein, administered to supraphysiological concentrations (1000 mg/kg feed) to rats did not cause significant toxicity to the female reproductive tract or provide a protective effect against chemically induced mammary cancer [Lamartiniere et al., 2002]. Response to doses of 500 mg/kg/day of genistein were related to the estrogenic properties, were reversible and no adverse effects were observed at 50 mg/kg/day [McClain et al., 2006]. Similarly, daidzein and SPI (with normal or low levels of isoflavones) were effective inhibitors of induced mammary tumors in adult rats [Constantinou et al., 2001]. Until now, it has been suggested that isoflavones themselves could have beneficial effects on cancer, cardiovascular disease, brain function, alcohol abuse, osteoporosis and menopausal symptoms but no effects on pancreas have been found. Therefore, despite the different dietary concentration of isoflavones on this study, the effects detected could not be attributed to the isoflavones since the values were 4 – 12 mg/kg feed for genistein and 8 – 20 mg/kg feed for daidzein

Even though the main antiphysiological or biologically active factors are absents or in concentrations with no negative effects, changes in pancreatic cells from rats fed on GM diet were detected by histology and gene expression evaluation. Typical

indicators of pancreatitis such as vacuolization or PMNs were not observed; however, acinar disorganization and zymogen granules depletion were similar to the symptoms of pancreatitis [Magaña-Gómez et al., 2006]. Furthermore, the depletion of zymogen granules detected in this study coincides with the findings of Malatesta et al. [2002b], who found a decreasing of total zymogen area in mice fed on GM diet after the first month. Interestingly, by the second month, the total zymogen area was significant increased in the same group, resembling a recuperation of the mouse acinar cell and a decrease appear later by the eight month. These changes are explained as reconstitution of the normal morphology and function of the pancreas. The pancreatic repair involves proliferation of cells from intact acini and tubular complexes [Lechene de la Porte et al., 1991]. Immunodetection of mouse acinar or duct cells in acinar culture cells showed that more than 97% of the labeled area was acinar positive 3 days after induced growth. It decreased to approximately 16% by day 9, and then returned to over 97% by day 21 of culture [De Lisle and Logsdon, 1990]. In these transitional stages between acinar and ductular cells, regular acinar cells are replete with dense zymogens granules, other cells contains less granules, intermediate cells might have no granules and final ductular cell is devoid of these [Bockman, 1997]. Furthermore, *de novo* acinar cells are empty of zymogens whereas mature cells should be replete. This process of differentiation and re-differentiation of pancreatic acinar cells can explain our finding of the highest score for zymogen granules depletion by the fifteen day and acinar disorganization by the fifth, as well as recovering after 30 days.

In these soy protein feeding bioassays, PAP mRNA induction appeared since the first day, before histological changes (zymogens granules depletion and acinar disorganization in pancreas) in the GM group pancreas. Differences in order of

magnitude between bioassays are explained by the use of different genes to normalize expression, since abundance of the ribosomal protein L13 mRNA is much lower than 18S rRNA, used in each bioassay, respectively. Additionally, rats in the first bioassay were four weeks old and at the second assay these were one week elder. Although the main function of PAP is still unclear, it has been proposed as an acute phase protein expressed in the defense reactions of pancreatic cells [Savkovic et al., 2004] that induces suppression of the local pancreatic, as well as systemic inflammation during acute pancreatitis [Paajanen et al., 1999]. Moreover, PAP increases in pancreatic cell injury without pancreatitis (subclinical cell damage) [Paajanen et al., 1999]. This could explain the increased abundance of PAP mRNA in GM group respect to Non-GM.

The quantification of trypsinogens mRNA in both bioassays, showed a parallel pattern to zymogen granules depletion shown in bioassay 1. Trypsinogens are regulated at transcriptional level [Iovanna et al., 1991, Lhoste et al., 1994]; thus, when there is no synthesis of zymogen, accumulation of their mRNA occurs until CCK stimuli evoke the protein synthesis and secretion of trypsinogens. Therefore, histological findings in rat pancreatic tissue as well as the effects on PAP and trypsinogens mRNA levels might suggest a cellular injury due to GM soy protein consumption.

The amylase levels show a gradual increase along the bioassay due to the postnatal functional maturation [Githens, 1990]. However, it was possible to observe slightly lower levels in the GM group respect to those of Non-GM. This result agree to the ones of Malatesta et al. [2002b] who found a lower amylase content in pancreatic tissue and lower labeling densities with anti-α-amylase antibody in acinar cells in GM soybean-fed mice than in controls. Therefore, it is possible that the stimulated synthesis

of trypsinogens related to the replenishment of the acinar cells exceeds the protein-synthesis capacity of the exocrine pancreas, thus decreasing synthesis of amylase.

The results appear to indicate that rats fed on GM diet, had a pancreatic supraphysiological stimuli or synergism with CCK; although it was not severe, it was sufficiently strong to induce a mild pancreatic injury with an adaptive response. In a long term feeding of GM soy diet, a progressive deleterious effect could be induced, ending in pancreatitis due to a long exposure to high level of endogenous CCK. However, it has been reported that CCK-58 is the only detectable endocrine form in the rat [Reeve et al., 2003]. In contrast to supramaximal CCK-8 or caerulein, acute or prolonged supraphysiological levels of endogenous CCK-58 do not cause pancreatitis [Yamamoto et al., 2007]; the response of rat pancreatic cell to continuous CCK stimulation is limited. In a model of continuous increasing rat pancreatic exocrine secretions [Miyasaka et al., 1992], CCK content of duodenal mucosa showed a phasic pattern with increased content on days 1, 3 and 14 and returning to the control level on day 30. In spite of this continuous stimulus, regenerative changes started on day 7 and were most prominent on day 14. The zymogen granules were decreased and recovered to normality by day 7. On day 14, the number of zymogen granules was almost normal. Furthermore, a down-regulation of the receptor CCK-A has been suggested as a protective mechanism against overstimulation [Ohlsson et al., 2000].

Thus, it is possible that some bioactive components different to lectin, protease inhibitors or isoflavones evoke a pancreatic response. These components could have been affected by the genetic modification itself [Novak et al., 2000]. It has recently demonstrated that transcriptional mRNA variants of the inserted construct occur on the GTS 40-3-2 expression for the EPSPS (the enzyme which characterize GTS 40-3-2 for

glyphosate resistance) [Rang et al., 2005]. Toxicity assays for biosafety assessment of EPSPS were done with the protein over-expressed in bacterial cells [Harrison et al., 1996]. Since mRNA variants were recently found, the definite proteins expressed in vegetable tissues might have suffered modification from gene rearrangements, and moreover, post-transcriptional and/or post-translational changes can not be ruled out and these, have not been evaluated.

The protein doses assayed maximized exposure without producing nutritional imbalance in weaning rats, as recommended in the guidelines for risk evaluation [Jonas et al., 1996]. Since the diet performance was similar between groups, it could be assumed that some as yet unidentified component of the GM soy protein isolate affected the pancreatic acinar cells, in a similar manner to pancreatopathy. Although it was a mild damage with fast recovery (30 days for rats), humans or other animals with different response to high levels of endogenous CCK or under regime of intermittent feeding with GM soy protein, could be differently affected. Finally, the analysis of mRNA levels to evaluate the stressing effects caused by genetically modified food are very sensitive protocols that can be used to evaluate this kind of effects in other mammals.

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Tables

Table 1

Composition of the experimental diets (g/kg)

Ingredient (g/kg)	NON-GM* 18% protein	GM† 18% protein
SUPRO 500E IP Non-GM soy protein isolate‡	204.5	---
SUPRO 500E GM soy protein isolate‡	---	204.5
DL-Methionine‖	3.0	3.0
Minerals mix¶	35	35
Vitamins mix**	10	10
Cellulose***	50	50
Corn oil	50	50
Choline Chloride***	2	2
Sucrose	500	500
Cornstarch	150	150

*NON-GM: Non-GM soy protein isolate diet

† GM: GM-soy protein isolate diet

‡ The Solae Company. St. Louis, MO. USA.

‖ Dyets Inc. Bethlehem, PA. USA.

¶AIN-76 mixture. Harlan Teklad, Madison, WI. USA.

** AIN-76A mixture. Harlan Teklad, Madison, WI. USA.

*** Harlan Teklad, Madison, WI. USA.

Table 2

Primers used for PCR and their characteristics

Primer name (Fw/Rv)	Nucleotide sequence (5' - 3')	Specificity	Product size (bp)	Reference
SL for SL rev	ATGGGCTTGCCTTCTTTCT CCGATGTGTGGATTTGGTG	Soybean lectin	157	[Germini et al., 2004]
CM01 CM02	CACTACAAATGCCATCATTGCGATA CTTATATAGAGGAAGGGTCTTGCGA	CaMVp35S	218	[Quist et al., 2001]
RR04 RR05	CCCCAAGTTCCTAAATCTTCAAGT TGCGGGCCGGCTGCTTGCA	CP4-EPSPS	180	[Studer et al., 1998]
Nostefor Nosterrev	GAATCCTGTTGCCGGTCTTGCG GCGGGACTCTAATCATAAAAACCC	NOS-terminator	125	
PAPfw PAPrv	TGAATTATGTCAACTGGGAGAGG TTACTGCTTTCCAAGACATGAGG	PAP	318	[Magaña-Gómez et al., 2006]
rTRY3/Fw rTRY3/Rv	TYTGTGGAGGYTCCCTCATC CGRGMATTGAGKKTSRCAGG	Trypsinogens	226	
rL13A/fw rL13A/rv	AAGCAGGTACTGCTGGG CCAACACCTTGAGGCGTT	L13A ribosomal protein	261	[Magaña-Gómez et al., 2006]
r18S/Fw r18S/Rv	GCAATTATTCCCCATGAACG AGTTCGACCGTCTTCTCAGG	18S rRNA	187	

Key to symbols: Y = C + T, R = A + G, M = A + C, K = G + T, S = G + C

Table 3

Content of protease inhibitors activity and isoflavones of Non-GM and GM- soy protein isolates (SPI)

	SPI 500E IP (Non-GM)	SPI 500E (GM)	Range in SPI	Reference
Trypsin inhibitor activity (mg TI/g protein)	4.36	4.84	1.4 – 29.4	[Anderson et al., 1995]
Chymotrypsin inhibitor activity (mg/g sample)	0.69	0.71	0.3 – 2.0	[Anderson et al., 1995]
Isoflavone (μg/g sample)				[USDA, 2002, Wang et al., 1994]
Genistein	52.6 ± 5.9*	25.9 ± 5.9	7.7 – 68.9	
Daidzein	92.9 ± 8.9*	49.2 ± 8.9	27.2 – 136	

Values are mean ± SE. The asterisk (*) signifies differences between soy protein isolates, $p < 0.05$.

Table 4

Growth and Nutritional Performance of rats fed GM or Non-GM diets

		Bioassay 1		Bioassay 2	
		GM Diet	**Non-GM Diet**	**GM Diet**	**Non-GM Diet**
Ingested food (g)	Day 3	26.3 ± 0.92	28.9 ± 0.92	36.3 ± 1.26	34.9 ± 1.26
	5	56.9 ± 2.14	57.9 ± 2.14	57.2 ± 1.12	60.9 ± 1.12
	15	201.2 ± 7.87	209.1 ± 7.87	197.2 ± 0.65	197.6 ± 0.65
	30	536.8 ± 13.8	524.2 ± 13.8	509.1 ± 2.9	507.4 ± 2.9
Weight gain (g)	Day 3	17.4 ± 0.59	16.5 ± 0.59	21.4 ± 1.21	21.9 ± 1.21
	5	27.1 ± 1.04	29.3 ± 1.04	37.3 ± 1.60	41.0 ± 1.60
	15	93.6 ± 3.41	93.9 ± 3.41	88.3 ± 1.49	91.5 ± 1.49
	30	192.6 ± 8.39	190.1 ± 8.39	191.7 ± 5.52	197.6 ± 5.52
Feed conversion*		0.36 ± 0.008	0.36 ± 0.008	0.38 ± 0.01	0.39 ± 0.01
Protein efficiency*		1.85 ± 0.04	1.85 ± 0.04	1.94 ± 0.05	1.99 ± 0.05

Values are mean ± SE. Analysis was made for each bioassay. (*) valued calculated from the group surviving at day 30. All values were not statistically different, $p > 0.05$.

Figures

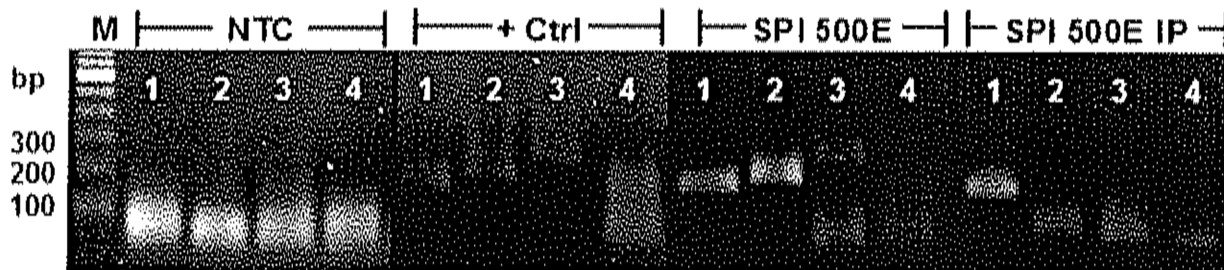


Fig. 1. Qualitative PCR analysis of soybean lectin, CP4-EPSPS, CaMV 35S and NOS terminator to characterize soybean samples.

M, PCR marker 100 bp DNA Ladder; NTC, no template control; + Ctrl, GM reference material; SPI 500E, soybean protein isolate 500E; SPI 500E IP, soybean protein isolate labeled as Non-GM. Lane 1, soybean lectin (157 bp); lane 2, CP4-EPSPS (180 bp); lane 3, CaMV p35S (218 bp); lane 4, NOS terminator (125 bp).

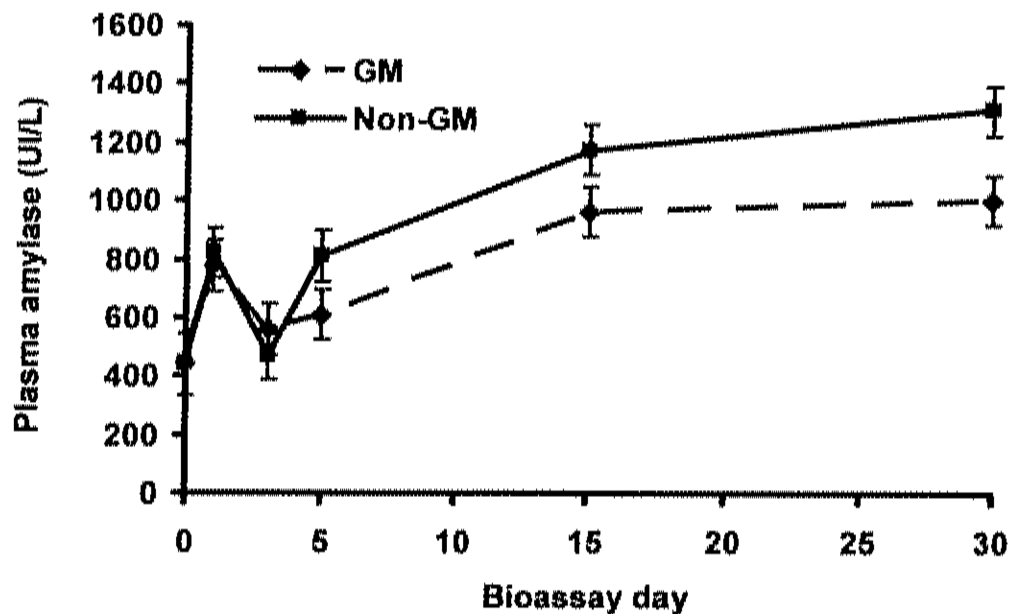


Fig. 2. Time course of plasma amylase of rats fed on Non-GM or GM diet.

Each point represents the mean ± S.E. No differences were found between diets, $p > 0.05$.

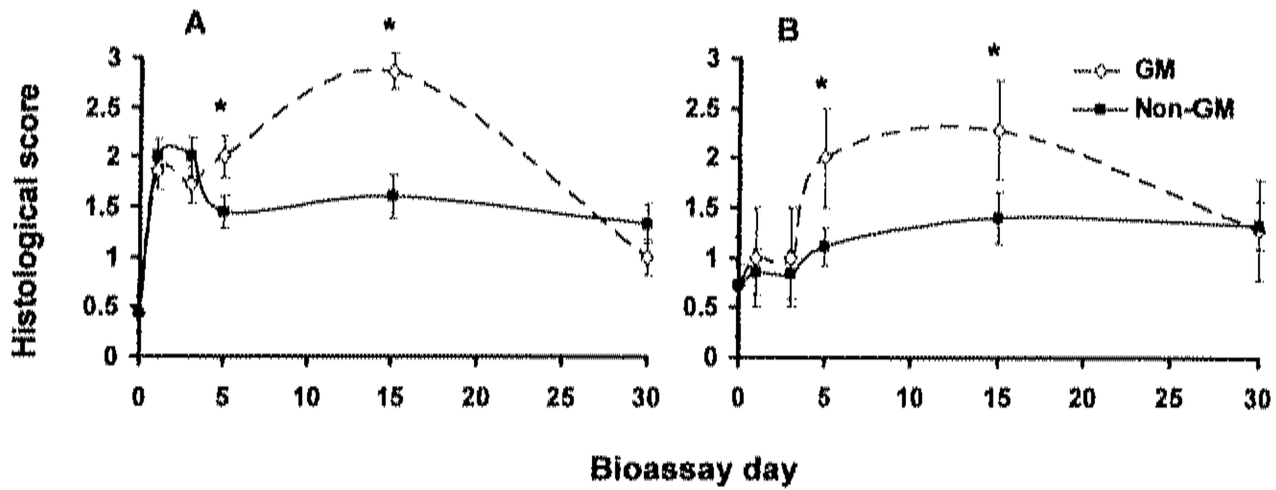
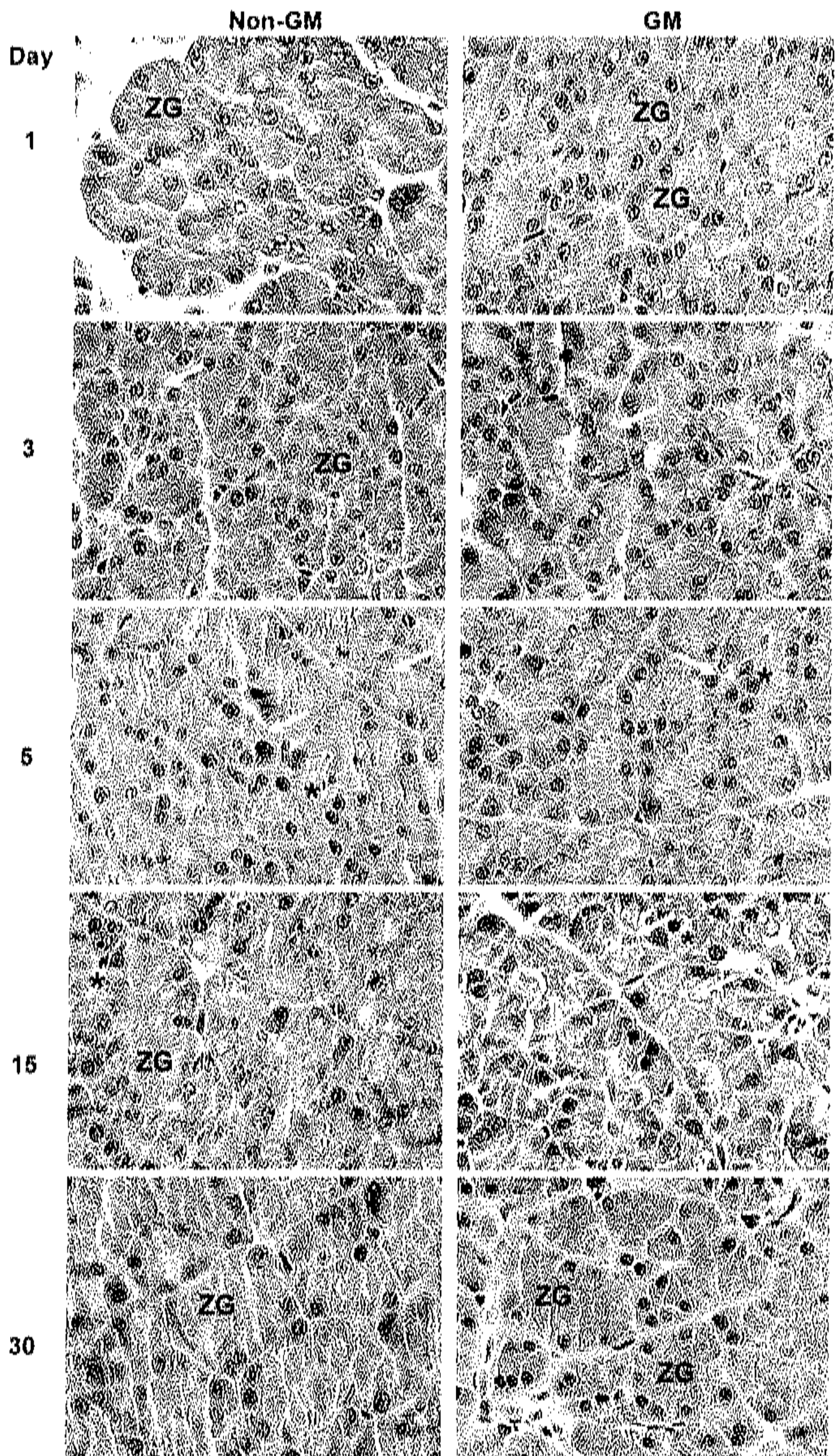


Fig. 3. Time course of histological changes in pancreas of rats fed on Non-GM or GM diet.

Zymogens granules depletion (A) and acinar disorganization (B). Each point represents the mean ± S.E. Asterisks show differences between diets, $p < 0.05$.



Non-GM
GM
Day
1
3
5
15
30
ZG
ZG
ZG
ZG
ZG
ZG
ZG
ZG

Fig. 4. Representative light-microscopic sections of rat pancreas fed on Non-GM or GM column soybean diet.

Sections show zymogens granules content (ZG, concentrated pink areas in the apical pole of the cells close to the acinar lumen) and acinar disorganization during the experimental time, according to diet. H & E staining (x132). Zymogen granule depletion (asterisk) and acinar disorganization (arrow) were different between diets in day five and fifteen ($p < 0.05$).

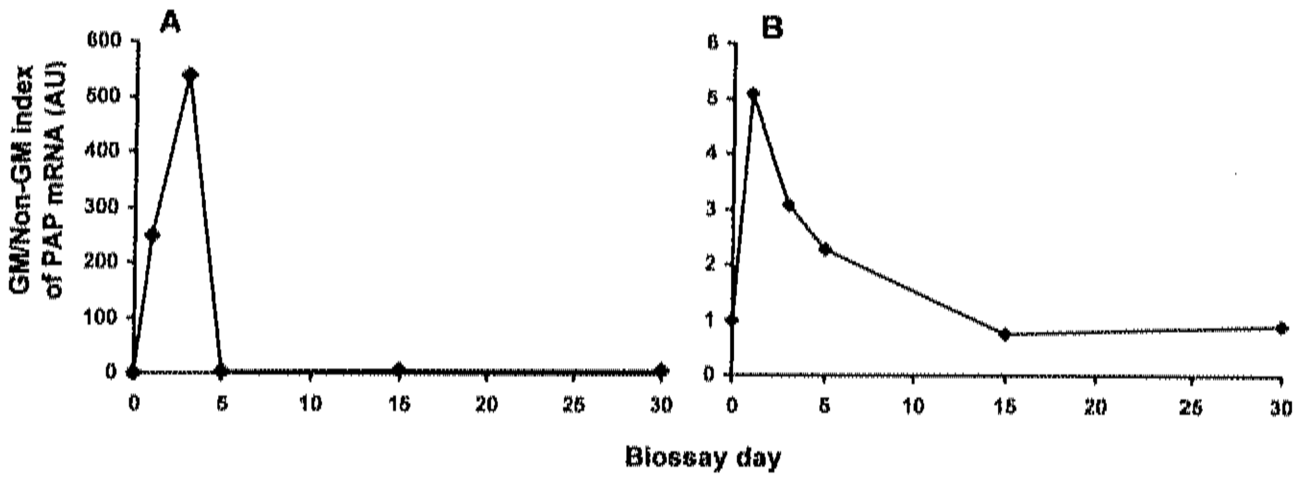


Fig. 5. GM/Non-GM index of relative quantification of PAP mRNA in pancreas of GM- and Non-GM-fed rats in biossay 1 (A) and 2 (B).

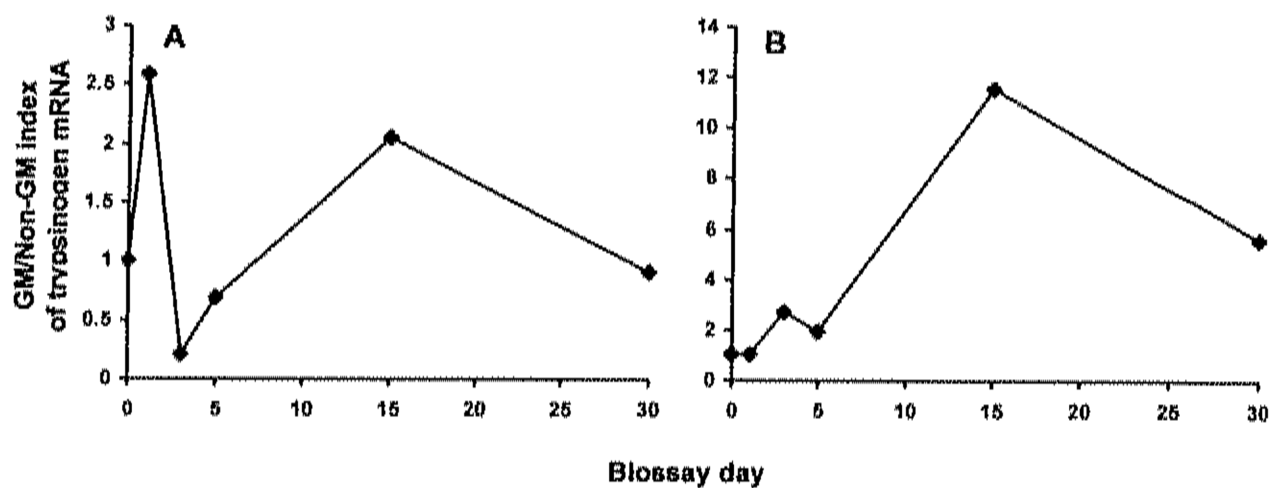


Fig. 6. GM/Non-GM index of relative accumulation of trypsinogens mRNA in pancreas of GM- and Non-GM-fed rats in biossay 1 (A) and 2 (B).