Histochem Cell Biol (2008) 130:967–977
DOI 10.1007/s00418-008-0476-x

ORIGINAL PAPER

A long-term study on female mice fed on a genetically modified soybean: effects on liver ageing

Manuela Malatesta · Federica Boraldi · Giulia Annovi · Beatrice Baldelli · Serafina Battistelli · Marco Biggiogera · Daniela Quaglino

Accepted: 1 July 2008 / Published online: 22 July 2008
© Springer-Verlag 2008

Abstract Liver represents a suitable model for monitoring the effects of a diet, due to its key role in controlling the whole metabolism. Although no direct evidence has been reported so far that genetically modified (GM) food may affect health, previous studies on hepatocytes from young female mice fed on GM soybean demonstrated nuclear modifications involving transcription and splicing pathways. In this study, the effects of this diet were studied on liver of old female mice in order to elucidate possible interference with ageing. The morpho-functional characteristics of the liver of 24-month-old mice, fed from weaning on control or GM soybean, were investigated by combining a proteomic approach with ultrastructural, morphometrical and immunoelectron microscopical analyses. Several proteins belonging to hepatocyte metabolism, stress response, calcium signalling and mitochondria were differentially expressed in GM-fed mice, indicating a more marked expression of senescence markers in comparison to controls. Moreover, hepatocytes of GM-fed mice showed mitochondrial and nuclear modifications indicative of reduced metabolic rate. This study demonstrates that GM soybean intake can influence some liver features during ageing and, although the mechanisms remain unknown, underlines the importance to investigate the long-term consequences of GM-diets and the potential synergistic effects with ageing, xenobiotics and/or stress conditions.

Keywords Ageing · Cell nucleus · Genetically modified soybean · Liver · Mitochondria

M. Malatesta (✉)
Dipartimento di Scienze Morfologico-Biomediche, Sezione di Anatomia e Istologia, University of Verona, strada Le Grazie 8, 37134 Verona, Italy
e-mail: manuela.malatesta@univr.it

F. Boraldi · G. Annovi · D. Quaglino
Department of Biomedical Sciences, University of Modena e Reggio Emilia, 41100 Modena, Italy

B. Baldelli · S. Battistelli
Istituto di Istologia e Analisi di Laboratorio, University of Urbino, 61029 Urbino (PU), Italy

M. Biggiogera
Dipartimento di Biologia Animale, Laboratorio di Biologia Cellulare e Neurobiologia, University of Pavia, 27100 Pavia, Italy

Introduction

Diet is considered one of the most important environmental factors affecting lifespan. Genetically modified (GM) crops, in which new genes have been inserted into the original genome, are nowadays distributed all over the world, thus frequently becoming part of human and animal diets (Sanvido et al. 2007). The fact that GM food may affect human or animal health is debated: actually, no consensus exists neither on the test designs nor on the criteria to be assumed for assessing the presence of possible pathological signs (Doull et al. 2007; Séralini et al. 2007). However, it cannot be ignored that some scientific reports have described structural and molecular modifications in different organs and tissues of GM-fed animals (e.g. Ewen and Pustzai 1999; Malatesta et al. 2002a, b, 2003a, 2005, Vecchio et al. 2004; Tudisco et al. 2006; Trabalza-Marinucci et al. 2008). These observations suggest that the risk of genetically modified crops cannot be ignored and deserves further investigations in order to identify possible long-term effects, if any, of GM food consumption that might help in the post market surveillance (Kuiper et al. 2004).

Within this context, it seems of prime importance to elucidate whether a GM-containing diet may interfere with the ageing process, since senescence is characterized by progressive changes in several cellular functions that eventually result in disease and/or the loss of the ability to successfully respond to stress and xenobiotics (Jameson 2004). In fact, to test GMO-related effects on laboratory mammals, experiments have usually been performed for some months only, thus making impossible to detect long-term consequences (Séralini et al. 2007).

Liver represents an especially suitable model for monitoring the effects of a diet, since it is a multifunctional organ exerting a key role in controlling the whole metabolism and in detoxifying toxic compounds. It is known that liver sensitivity to xenobiotics is gender-related, many detoxification pathways being hormone-regulated (e.g. Voss et al. 2003); for this reason, our studies were carried out only on female animals, which seem to be more capable to cope with exogenous stress conditions (e.g. Lin et al. 2003; Dai et al. 2006; Patel et al. 2008).

In previous studies on hepatocytes from young and adult (2–8 months of age) female mice fed on GM soybean we demonstrated nuclear modifications involving structural constituents of the transcription and splicing processing pathways (Malatesta et al. 2002a).

In the present study, we have investigated the morpho-functional characteristics of the liver of 24-month-old female mice, fed from weaning on control or GM soybean, by combining a proteomic approach with ultrastructural, morphometrical and immunocytochemical analyses.

Materials and methods

Animals and treatments

Ten female Swiss mice were fed on a laboratory chow (Malatesta et al. 2002a) containing 14% GM soybean, a percentage corresponding to that usually present in the standard diet of this mouse strain and also included in the range (11–33%, Séralini et al. 2007) generally used in the regulatory tests for GMOs. This GM soybean has been obtained by insertion of the bacterial CP4 EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) gene conferring a high level of tolerance to glyphosate, the active ingredient of the herbicide Roundup (GTS 40-3-2; Padgette et al. 1995). In parallel, ten female mice were fed on the same diet with commercial, non-GM soybean (controls). Both animal groups started their respective diets at weaning and were grown in standard cages under constant environmental conditions (21 ± 1°C, 50 ± 5% moisture, 12L:12D daylight cycle) until 24 months of age. During treatment, mice were weighed every 2 months. After death by cervical dislocation, liver was quickly removed, weighed and the right lobe of the organ was partly processed for microscopical analyses, and partly stored in liquid nitrogen for proteome analysis.

All animals received humane care and this study protocols comply with the institution's guidelines.

Proteome analysis

Sample preparation Liver samples were obtained from three mice for each experimental condition and were kept separate during all experiments. The tissue was homogenized and immediately resuspended in lysis buffer (8 M urea, 2% CHAPS, 65 mM dithioerythritol, 2% pharmalyte pH 3–10 and trace amount of bromophenol blue). Protein concentration was determined according to Bradford (1976).

2-DE 2-DE was performed in two independent assays, where samples from all animals were run in triplicate. Samples containing 60 µg (analytical gels) or 1 mg (preparative gels) of protein underwent 2-DE using the Immobiline/polyacrylamide system (Bjellqvist et al. 1993). Isoelectric focusing was performed on IPGphor system (GE-Healthcare, Uppsala, Sweden) at 16°C using two different protocols. For analytical gels: passive rehydration for 16 h, 500 V for 1 h, 500–2,000 V for 1 h, 3,500 V for 3 h, 5,000 V for 30 min and 8,000 V for 12 h. For preparative gels a preliminary step at 200 V constant for 12 h was added. Thereafter, immobilized pH gradient strips were reduced (2% dithioerythritol) and alkylated (2.5% iodoacetamide) in equilibration buffer (6 M urea, 50 mM Tris–HCl, pH 6.8, 30% glycerol, 2% SDS). Subsequently, strips were loaded onto 12% acrylamide vertical gels using an Ettan DALTsix electrophoresis unit (GE-Healthcare, Uppsala, Sweden). Analytical gels were stained with ammoniacal silver nitrate (Hochstrasser et al. 1988), whereas preparative gels for mass spectrometric analysis were silver-stained (Shevchenko et al. 1996).

Data acquisition and analysis To detect significant differences in protein abundance between the two experimental conditions, all silver-stained gel images were digitalized at 400 dpi resolution using ImageScanner (GE-Healthcare, Uppsala, Sweden) and analysed using Melanie 3.0 software (GE-Healthcare, Uppsala, Sweden). After background subtraction, protein spots were automatically defined and quantified with the feature detection algorithm (Hochstrasser et al. 1988). Spot intensities were expressed as percentages (vol %) of relative volumes by integrating the optical density (OD) of each pixel in the spot area (vol) and dividing with the sum of volumes of all spots detected in the gel. Only those spots, within the same experimental condition, exhibited the same trend of

expression in all gels underwent further quantitative analysis. Mean values, standard deviations and coefficients of variation were calculated. Statistical data were obtained using GraphPad software (San Diego, CA, USA) and compared by the unpaired *t* test. Differences between treatments were considered significant at $P < 0.05$. For MS analysis only those spots whose expression appeared significantly changed upon GM soybean treatment have been selected.

In-gel destaining and digestion of protein samples Spots of interest were manually excised from preparative silver-stained 2-DE gels. Silver-stained gel pieces were destained as described by Gharahdaghi et al. (1999). All excised spots were incubated with 12.5 ng/µl sequencing grade trypsin (Roche Molecular Biochemicals, Basel, Switzerland) in 25 mM AmBic overnight at 37°C. Peptide extraction was carried out twice using 50% ACN, 1% TFA and then 100% ACN. All extracts were pooled, and the volume was reduced by SpeedVac.

Mass spectrometry

Peptides were resuspended in aqueous 5% formic acid and subsequently eluted onto a 150 mm × 75 µm Atlantis C18 column analytical (Waters, Milford, MA, USA) and separated with an increasing ACN gradient from 10 to 85% in 30 min using a Waters CapLC system. The analytical column (estimated flow approximately 200 nL/min) was directly coupled, through a nanoES ion source, to a Q-TOF Ultima Global mass spectrometer (Waters, Milford, MA, USA). Multicharged ions (charge states 2, 3 and 4) were selected for fragmentation and the acquired MS/MS spectra were searched against the SWISS-PROT/TrEMBL non-redundant protein and NCBI database using the Mascot (www.matrixscience.com) MS/MS search engine. Initial search parameters were the follows: enzyme, trypsin; maximum number of missed cleavages, 1; fixed modification, carbamidomethylation of cysteines; variable modification parameters, oxidation Met; peptide tolerance, 0.5 Da; MS/MS tolerance, 0.3 Da; charge state, 2, 3, or 4. We basically selected the candidate peptides with probability-based MOWSE scores that exceeded its threshold, indicating a significant (or extensive) homology ($P < 0.05$), and referred to them as "hits". The criteria were based on the manufacturer's definitions (Matrix Science, Boston, MA, USA) (Honore et al. 2004). Proteins identified with at least two peptides were validated without any manual processing, when score higher than 40, whereas were systematically checked and/or interpreted manually to confirm or cancel MASCOT suggestions, when score was lower than 40 but higher than 20.

Light microscopy

For conventional histological observations, liver samples were fixed by immersion in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 6 h at 4°C, then dehydrated with ethanol and embedded in paraffin. Five-µm-thick sections were stained with either haematoxylin–eosin or Mallory's connective tissue stain. Samples were observed in an Olympus BX51 light microscope.

Electron microscopy

For conventional ultrastructural morphology and mitochondria morphometrical evaluations, liver samples were fixed with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M Sörensen phosphate buffer, pH 7.4 for 3 h, washed, post-fixed with 1% OsO_4 at 4°C for 1 h, dehydrated and embedded in Epon.

For morphometrical and immunocytochemical studies on cell nuclei, samples were fixed with 4% paraformaldehyde in 0.1 M Sörensen buffer at 4°C for 2 h, dehydrated and embedded in LRWhite resin.

Epon-embedded ultrathin sections were conventionally contrasted with uranyl acetate and lead citrate, while LRWhite-embedded sections were stained with the EDTA method (Bernhard 1969). This procedure entails three steps: first, the sections are contrasted with uranyl acetate, then they are exposed to EDTA to bleach condensed chromatin, whose contrast generally masks the ribonucleoprotein (RNP) constituents involved in RNA processing, and, finally, lead citrate is briefly applied to increase RNP contrast. Specimens were observed in a Philips Morgagni TEM equipped with a Megaview II camera for digital image acquisition.

Morphometry

Morphometrical analyses were carried out by using the AnalySIS Image processing software (Soft Imaging System GmbH, Germany). Cellular and nuclear areas were measured (440×) on 30 hepatocytes/animal; then, the nucleus/cytoplasm (N/C) ratio was calculated. Further morphometrical evaluations (11,000×) were made on ten hepatocyte nuclei/animal: nucleolar areas, percentages of fibrillar centres (FCs), dense fibrillar component (DFC) and granular component (GC) per nucleolus, FC area, index of nuclear shape irregularity (the ratio between the measured perimeter and the circumference of the equivalent circle), percentage of nuclear area occupied by condensed chromatin, perichromatin granule density (PG/µm^2 of nucleoplasm) and nuclear pore frequency (NP/µm of perimeter) were considered. Finally, the sectional area as well as inner and outer membrane profile length were

measured (18,000×) in 20 mitochondria/animal, then the inner/outer membrane length ratio was calculated in order to estimate the inner membrane length independently from mitochondrial size.

Immunoelectron microscopy

For immunocytochemical analyses, mouse monoclonal antibodies directed against phosphorylated polymerase II (Research Diagnostics Inc., Flanders, NJ, USA) and the splicing factor SC-35 (Sigma-Aldrich, Buchs, Switzerland) were used and revealed by secondary gold-conjugated probes (Malatesta et al. 2002a, b). Labelling density (number of gold grains/μm^2) over nucleoplasm and nucleolus was evaluated on ten nuclei/animal (18,000×). Areas of interest were measured as described above and gold grains counted manually.

Data for each variable were pooled according to the experimental group and expressed as mean ± standard error (SE). Statistical comparisons were performed by the one-way ANOVA test ($P \leq 0.05$).

Results

Mice body weight was quite similar in all animals during the whole experiment. At sacrifice, animal's weight varied from 21 to 29 g, whereas liver's weight ranged from 0.6 to 1.5 g, without significant differences between control and GM soybean-fed animals. No macroscopic alterations or pathologic lesions were observed in any organ of all animals.

Proteomics

The total protein content of the liver did not reveal any significant difference between control and GM-fed mice, as evaluated by the Bradford assay. Similarly, the number of proteins separated by 2-DE was approximately of 1,400 from each sample, independently from the experimental condition.

In order to exclude the influence of possible high intra-sample variability, the coefficient of variation (CV) (standard deviation of normalized spot volume divided by mean) was evaluated for each sample from triplicate parallel preparations.

As reported (Molloy et al. 2003), we considered for further analysis spots with CV values of normalized volumes lower than 30%. Consistently with the observation that the great majority of proteins gave reproducible results in terms of sample preparation, extraction procedures and 2-DE, CV values of normalized volumes higher than 30% were only obtained for very faint spots and for spots located close to the gel edges.

In the liver of GM-fed mice we demonstrated significant changes in the expression of 49 spots, as indicated by labels on two representative gels obtained from control (Fig. 1a) and GM-fed animals (Fig. 1b). In particular, 39 proteins appeared significantly more expressed in GM-fed mice, whereas 10 proteins were significantly decreased.

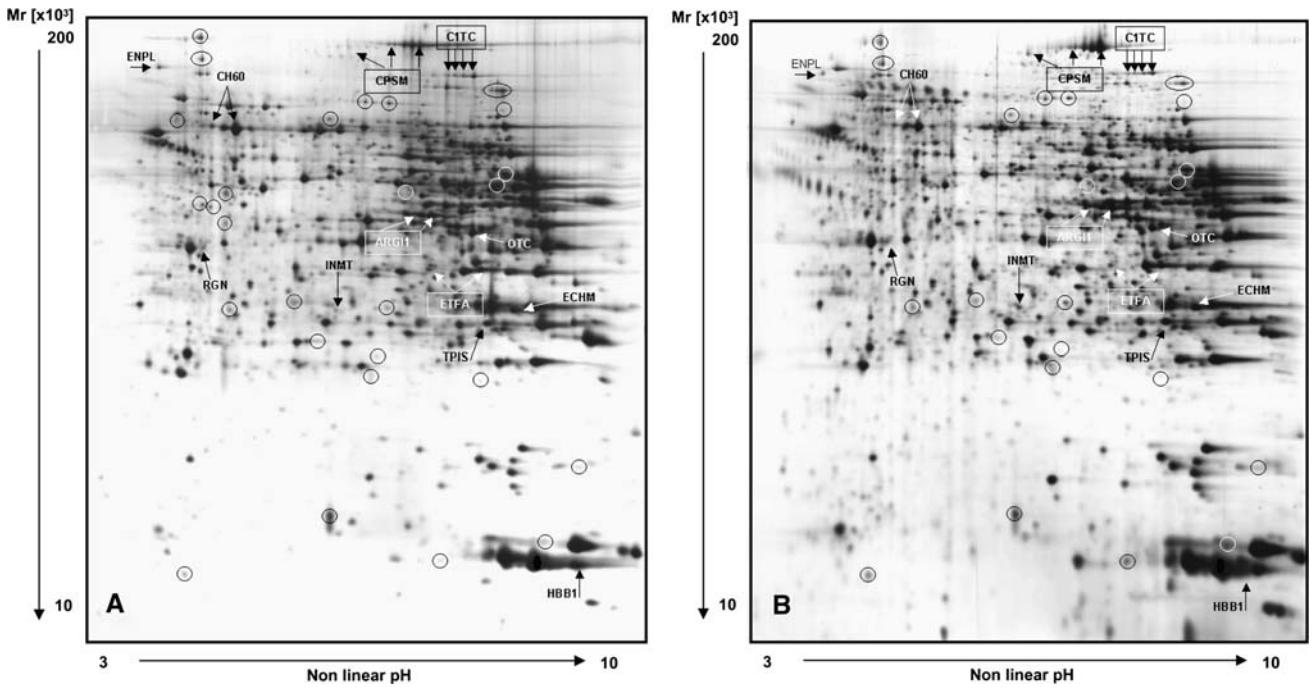

Fig. 1 Representative silver-stained 2-D electropherograms of liver from control (**a**) and GM-fed mice (**b**). Differentially expressed proteins are indicated by *arrows* and *symbol name* when identified, or by *open circles* when unidentified

By mass spectrometry we identified 20 differentially expressed proteins in the whole tissue lysate (Table 1). The remaining proteins were either in insufficient amount to be analysed by MS or MS/MS or the MS-compatible staining procedure failed to reveal them.

Protein distribution into functional categories indicates that the majority of differentially expressed proteins identified by MS belong to hepatocyte metabolism (namely, nitrogen, carbohydrate and lipid metabolism), stress response and calcium signalling pathways, as well as to mitochondria.

Morphology

The general structure of the liver parenchyma showed similar organization of hepatocytes, blood vessels, bile ducts, and extracellular matrix in the periportal areas in control and GM soybean-fed mice.

Accordingly, hepatocyte cytoplasmic organelles showed similar features in all animals: abundant rough and smooth endoplasmic reticulum, well-developed Golgi apparatus, ovoid mitochondria with transversal cristae, glycogen deposits, lipid droplets and some residual bodies (not shown).

Conversely, hepatocyte nuclei of GM-fed mice showed some morphological differences in comparison to controls (Fig. 2a, b). Hepatocyte nuclei from control mice showed a roundish shape characterized by little irregularities appearing as a fine waving and contained clumps of condensed chromatin distributed both at the periphery and inside the nucleus. In the nucleoplasm, perichromatin fibrils (PF) and perichromatin granules (PG) were distributed along the borders of the condensed chromatin, while interchromatin granules (IG) occurred as clusters in the interchromatin space (Fakan 2004). Nucleoli exhibited easily recognizable, intermingled, dense fibrillar (DFC) and granular components (GC), whereas fibrillar centres (FC) were not prominent (Fig. 2a) (nucleolar nomenclature in Schwarzacher and Wachtler 1993). Hepatocyte nuclei from GM-fed mice differed from controls because of more regular contour, large clumps of condensed chromatin, high number of PG, and smaller and compact nucleoli rich in GC (Fig. 2b). Interestingly, many PG were observed inside the condensed chromatin areas.

Morphometry

Cellular and nuclear areas were generally smaller in GM-fed than in control mice, without modifying the N/C ratio.

Table 1 Differentially expressed proteins identified in the liver of GM-fed mice compared to controls

Symbol[a]	Protein name and accession number	Theoretical MW(kDa)/p*I*	Fold-change ± SD[b]	Identification method, % coverage, no. matched peptides
ARGI1	Arginase-1 (EC 3.5.3.1) (Q61176)	34.8/6.52	+2.1 ± 0.5*	MS/MS, 49%, 10
ARGI1	Arginase-1 (EC 3.5.3.1) (Q61176)	34.8/6.52	+1.6 ± 0.3*	MS/MS, 38%, 8
C1TC	C-1-tetrahydrofolate synthase, cytoplasmic (EC 1.5.1.5) (Q922D8)	101.2/6.68	+3.6 ± 1.0*	MS/MS, 10%, 9
C1TC	C-1-tetrahydrofolate synthase, cytoplasmic (EC 1.5.1.5) (Q922D8)	101.2/6.68	−1.8 ± 0.4*	MS/MS, 2%, 3
C1TC	C-1-tetrahydrofolate synthase, cytoplasmic (EC 1.5.1.5) (Q922D8)	101.2/6.68	−3.4 ± 1.1*	MS/MS, 5%, 5
C1TC	C-1-tetrahydrofolate synthase, cytoplasmic (EC 1.5.1.5) (Q922D8)	101.2/6.68	−4.4 ± 1.3**	MS/MS, 5%, 5
CH_60	60 kDa heat shock protein, mitochondrial (P63038)	60.9/5.91	−2.0 ± 0.5*	MS/MS, 34%, 13
CH_60	60 kDa heat shock protein, mitochondrial (P63038)	60.9/5.91	+1.9 ± 0.3*	MS/MS, 43%, 15
CPSM	Carbamoyl-phosphate synthase (EC 6.3.4.16) (Q8C196)	164.6/6.48	+ 3.1 ± 1.1**	MS/MS, 17%, 21
CPSM	Carbamoyl-phosphate synthase (EC 6.3.4.16) (Q8C196)	164.6/6.48	+2.1 ± 0.7*	MS/MS, 6%, 2
CPSM	Carbamoyl-phosphate synthase (EC 6.3.4.16) (Q8C196)	164.6/6.48	+2.6 ± 1.0*	MS/MS, 11%, 16
ETFA	Electron transfer flavoprotein subunit alpha, mitochondrial (Q99LC5)	35/8.62	−2.0 ± 0.3*	MS/MS, 18%, 4
ETFA	Electron transfer flavoprotein subunit alpha, mitochondrial (Q99LC5)	35/8.62	−2.2 ± 0.5*	MS/MS, 5%, 1
ENPL	Endoplasmin (P08113)	92.4/4.74	−2.0 ± 0.6*	MS/MS, 7%, 6
ECHM	Enoyl-CoA hydratase, mitochondrial (E C 4.2.1.17) (Q8BH95)	31.5/8.76	+1.9 ± 0.2*	MS/MS, 4%, 1
HBB1	Haemoglobin subunit beta-1 (P02088)	15.8/7.13	+2.0 ± 0.6*	MS/MS, 29%, 4
INMT	Indolethylamine N-methyl-transferase (EC 2.1.1.49) (P40936)	29.5/6	+2.1 ± 0.9*	MS/MS, 5%, 1
OTC	Ornithine carbamoyl-transferase, mitochondrial (EC 2.1.3.3) (P11725)	39.8/8.81	−3.2 ± 1.1**	MS/MS, 29%, 9
RGN	Regucalcin (Q64374)	33.4/5.16	−2.3 ± 0.6*	MS/MS, 38%, 9
TPIS	Triosephosphate isomerase (EC 5.3.1.1) (P17751)	26.7/6.9	−2.2 ± 0.9*	MS/MS, 40%, 5

[a] Symbols correspond to the entry name of the sequence and are reported on gels in Fig. 1

[b] Data represent the mean fold change variation ("+" increase and "−"decrease) in GM-fed mice versus control animals. * $P < 0.05$, ** $P < 0.01$

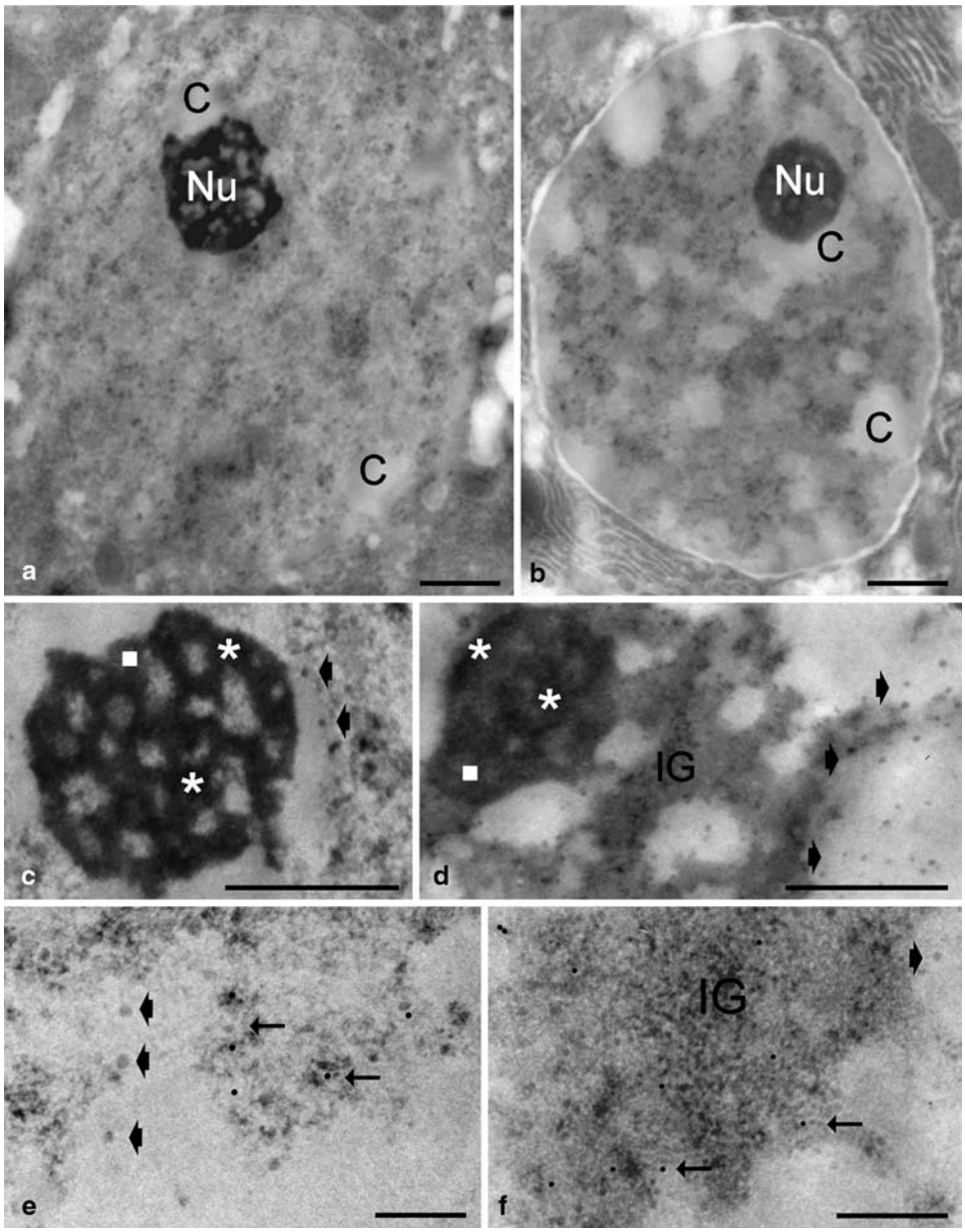

Fig. 2 Hepatocyte nuclei from a control mouse (**a**, **c**) and from a GM-fed mouse (**b**, **d**); LRWhite-embedded EDTA-stained samples. The general aspect of the nucleus is similar; however, in GM-fed mice the condensed chromatin (**c**) is quite abundant, PGs (*arrowheads*) are very numerous and occur also inside condensed chromatin areas, the nucleoli (*Nu*) appear smaller and more compact. *Asterisks*: DFC; *squares*: GC; IG: interchromatin granules. *Bars*: 1 μm. **e** Immunolabelling with anti-polymerase II antibody; the signal is specifically located on pericromatin fibrils (*arrows*), whereas PG (*arrowheads*) are devoid of labelling. **f** Immunolabelling with anti-SC-35 antibody; the gold grains occur on perichromatin fibrils (*arrows*) and on interchromatin granules (*IG*). The *arrowhead* indicates an unlabelled PG. *Bars*: 0.2 μm. The gold grain contrast was digitally enhanced using Adobe Photoshop

In GM-fed mice, shape index, nuclear pore frequency and nucleolar area decreased, while condensed chromatin percentage, PG density and GC percentage significantly increased (Table 2).

In mitochondria of GM-fed mice, a significant decrease in the inner/outer membrane length ratio (2.35 ± 0.05 vs. 3.19 ± 0.04, respectively, $P < 0.001$) was observed, although the mitochondrial area remained unchanged (0.23 ± 0.01 μm^2 vs. 0.27 ± 0.01 μm^2, respectively, $P = 0.199$).

Immunoelectron microscopy

No difference in the localization of polymerase II and SC-35 was found between GM-fed and control mice. As expected, polymerase II was mainly associated with PF (Fig. 2e), while SC-35 specifically occurred on PF and IG (Fig. 2f). By contrast, quantitative evaluation of immunolabelling revealed a significantly weaker labelling for polymerase II and SC-35 in GM-fed mice (Table 3).

Discussion

The present study was performed to investigate the effects on female mouse liver of a 2-year-long diet containing 14% GM soybean and its potential impact on the physiological ageing process; this is, to our knowledge, the longest test so far performed on laboratory mammals fed on a commercially available GMO.

Table 2 Mean ± SE values of variables considered in hepatocytes of the two groups of animals

	Cell area	Nuclear area	N/C ratio	Shape index	Condensed chromatin (%)	PG density	Pore frequency	Nucleolar area	FC area	FC (%)	DFC (%)	GC (%)
Control	405.77 ± 13.86*	45.14 ± 1.95*	0.11 ± 0.001	1.38 ± 0.07*	27.55 ± 1.44*	0.76 ± 0.04*	0.69 ± 0.03*	2.25 ± 0.13*	0.05 ± 0.01	6.92 ± 0.71	29.65 ± 1.35	63.48 ± 1.28*
GM-fed	291.26 ± 9.42*	36.95 ± 1.42*	0.13 ± 0.006	1.19 ± 0.02*	41.05 ± 1.06*	1.73 ± 0.06*	0.55 ± 0.02*	1.70 ± 0.10*	0.05 ± 0.01	5.40 ± 0.90	25.63 ± 0.98	68.97 ± 1.41*
P values	<0.001	0.05	0.071	0.02	<0.001	<0.001	0.002	0.007	0.877	0.285	0.060	0.022

DFC dense fibrillar component; *FC* fibrillar centre; *GC* granular component; *N/C* nucleus/cytoplasm; *PG* perichromatin granule

The effect of gender on hepatic metabolism has been extensively examined for a number of drugs and xenobiotics, and female rats seem to be less sensitive to many xenobiotic treatments as demonstrated for DCPT (Patel et al. 2008), APAP (Dai et al. 2006, 2008), or clivorine (Dai et al. 2006, 2003, 2008). Gender-associated variations may be related to differences in the hepatic transport (Torres 1996), in the fluidity of the liver sinusoidal membranes (i.e. phosphatidylethanolamine to phosphatidylcholine ratio), and in higher levels of total *mdr* gene products in female rat livers compared to males (Morris et al. 2003). It has also been suggested that differences in the hepatic antioxidant defence mechanisms could be responsible for the higher resistance of females to some hepatotoxicants (Sverko et al. 2004; Justo et al. 2005). Other gender differences are hormone-dependent: i.e. the arginine metabolism (Kumar and Kalyankar 1984; Tipton 2001) and the expression of heat shock proteins (HSPs) (Voss et al. 2003) are related to the estrogen availability. By considering these all, we carried out our experiments only on female mice.

We performed a complementary interdisciplinary approach which allowed us to reveal changes in liver protein profile as well as in structural cellular components that may suggest morpho-functional changes in the organ.

The macroscopical analysis did not reveal evident differences between control and GM-fed mice; moreover, no significant difference in the mortality rate was observed between the two animal groups. The liver weight reduction observed, independently from treatment, in old animals compared to young and adult mice (Malatesta et al. 2002a) is a well-known phenomenon occurring during ageing (Schmucker 1990; Anantharaju et al. 2002).

Proteome analysis demonstrated in GM-fed mice a differential expression of a number of proteins mostly related to metabolic pathways (i.e. lipid and carbohydrate) and to the urea cycle. In particular, as for the urea cycle, arginase and carbamoyl-phosphate synthetase were significantly increased, whereas ornithine transcarbamoylase was markedly downregulated. Arginase is a cytosolic enzyme responsible for the cleavage of arginine, an amino acid particularly required during growth, stress and injury acting, and also involved in the metabolism of biologically active compounds (Tong and Barbul 2004). Arginine cleavage generates urea and ornithine, the latter being necessary for tissue repair processes (Witte and Barbul 2003; Durante et al. 2007) as well as for citrullin production in the mitochondrial matrix, where ornithine transcarbamoylase catalyzes the condensation of ornithine with carbamoyl phosphate.

Since nitric oxide (NO) synthase and arginase compete for arginine, it could be speculated that increased expression of arginase might be associated to a decrease in NO synthase activity, thus influencing the redox grading which

Table 3 Mean ± SE values of labelling densities obtained with anti-RNA polymerase II, and anti-SC35 antibodies on hepatocyte nuclei of the two animal groups. Background: 0.14 ± 0.01 gold grains/μm^2

	Anti-pol II nucleoplasm	Anti-pol II nucleolus	Anti-SC35 nucleoplasm	Anti-SC35 nucleolus
Control	1.15 ± 0.10	0.31 ± 0.08	2.52 ± 0.10	0.31 ± 0.08
GM-fed	0.74 ± 0.06	0.24 ± 0.06	2.01 ± 0.08	0.28 ± 0.07
P values	<0.001	0.576	<0.001	0.815

contributes to dual activation of proliferating and proapoptotic cascades (Carreras and Poderoso 2007), ultimately modulating senescence (Gilca et al. 2007). Consistently, it is known that NO synthase activity undergoes reduction with increasing age (Valdez et al. 2004; Numao et al. 2007). Interestingly, NO synthase also occurs in the inner mitochondrial membrane, functioning as a regulatory factor of respiration (Kato and Giulivi 2006), and reduced mitochondrial inner membrane as well as decreased expression of some respiratory enzymes have been found in GM-fed mice.

In GM-fed mice, we also found a significant downregulation of the senescent marker regucalcin. Regucalcin plays a regulatory role in intracellular signalling systems (Yamaguchi 2005) maintaining intracellular Ca(2+) homeostasis through activation of Ca(2+) pump enzymes in plasma membrane, endoplasmic reticulum and mitochondria, beside its activatory effect on SOD in the liver cytosol (Fukaya and Yamaguchi 2004). A reduced expression of regucalcin could imply an impaired ratio between pro-oxidant and anti-oxidant molecules. It is known that regucalcin levels significantly decrease in aged rats, thus favouring the age-dependent deterioration of liver (Tobisawa et al. 2003; Fujita 1999). It seems therefore that senescence pathways are significantly activated in GM-fed mice.

Furthermore, proteome analysis revealed a downregulation of several HSPs in GM-fed animals. HSPs are expressed in response to a wide variety of physiological and environmental insults, acting as molecular chaperones for nascent and stress-accumulated misfolded proteins, or mediating immunological functions, thus exerting a protective function (Multhoff 2006; Schmitt et al. 2007). However, it is known that during ageing a reduction in the expression of HSPs occurs (e.g. Rea et al. 2001; Zhang et al. 2002), thus contributing to the lower capability of elderly to cope against xenobiotics and stress conditions. Again, hepatocytes of GM-fed animals seem to be characterized by a more pronounced senescence than controls.

Several findings also suggest a lower metabolic activity in hepatocytes from GM-fed mice in comparison to controls. First, smaller cellular and nuclear areas, without alteration of the N/C ratio, generally indicate decreased metabolic rate (Hildebrand 1980). When compared to previous data (Malatesta et al. 2002a, 2005), cellular and nuclear areas from both control and GM-fed old mice show dimensions more similar to young than adult animals, according to previous data demonstrating an increase in size during development and maturation and a decline during ageing (Schmucker 1990). Nucleoli also decrease in size and show prominent GC, suggesting a downregulation of ribosomal RNA transcription/processing and export rate (Schwarzacher and Wachtler 1993). In addition, more regular nuclear contour and lower nuclear pore frequency imply reduced nucleus–cytoplasmic molecular trafficking (e.g. Malatesta et al. 1998); weaker labelling for RNA polymerase II and SC-35, and higher amounts of condensed chromatin indicate reduced transcriptional activity; higher densities of PG, site of intranuclear storage and transport of already spliced (pre)-mRNA (Fakan 2004), suggest altered pre-mRNA processing and/or impaired intranuclear or nucleus–cytoplasmic transport (e.g. Lafarga et al. 1993).

Interestingly, most of these findings represent a peculiar response of aged GM-fed animals; in fact, until the 12th month of age, hepatocyte nuclei of GM-fed mice show features typical of high metabolic rate (Malatesta et al. 2002a, 2005). It is known that a general decrease in transcription and splicing factors (Frasca et al. 2003; Malatesta et al. 2003b, 2004), in nucleocytoplasmic transport factors (Pujol et al. 2002) and in nuclear pore number (Galy et al. 2000) occur in hepatocyte nuclei during ageing, together with a PG accumulation (Malatesta et al. 2003b). However, in old mice fed on GM soybean these age-related alterations appear to be more pronounced than in controls. It could be hypothesized that the high metabolic rate observed in younger mice fed on GM soybean may accelerate the ageing process, possibly favouring the accumulation of reactive oxygen species (ROS) (e.g. Hallen 2002; Roijkind et al. 2002), thus contributing to the expression of senescent markers.

At present, we do not know which could be the factor(s) present in the GM soybean capable of inducing such modifications. The changes of regucalcin as well as of other differentially expressed liver proteins observed in the present study are comparable to that reported after exposure of several xenobiotics (Yamaguchi et al. 2002; Pastorelli et al. 2006; Wei et al. 2008), thus suggesting the involvement of similar pathways activated in response to different toxic compounds. The soybean used in this study has been

treated in the field with the herbicide Roundup; although the treatment conditions used were not specified by the manufacturer, it is worth considering the possible presence in the chow of traces of glyphosate (Granby et al. 2003), i.e. the active ingredient of the herbicide Roundup to which the soybean has been rendered tolerant (Padgette et al. 1995). It has been demonstrated that Roundup slows down transcription (Marc et al. 2005), interferes with estrogen synthesis (Richard et al. 2005) and depresses respiratory activity (Peixoto 2005), inducing alterations of the mitochondria inner membrane (Szarek et al. 2000). Although the respiratory activity of mitochondria declines during ageing (Schmucker 1990), in GM-fed old mice such a decrease appeared to be significantly more pronounced than in controls. Again, this phenomenon becomes evident only in aged animals: in fact, measurements performed on 8 and 12-month-old mice revealed no modification of mitochondrial membrane length (unpublished results).

At present, no data are available on the effects of this GM-containing diet on old male mice, but microscopical observations on livers of 3-month-old male mice (unpublished results) revealed a situation comparable to that found in females of the same age (Malatesta et al. 2002a), thus suggesting a limited influence of the gender on the effects of this GM soybean. On the other hand, the comparison of the features of hepatocytes from young and old GM-fed female mice seems indicate the occurrence of cumulative long-term effects: GM soybean would first enhance liver metabolism, and this prolonged activation may then accelerate the ageing process with increased expression of senescent markers.

In conclusion, the present work demonstrate that GM soybean intake can influence the liver morpho-functional features during the physiological process of ageing and, although the mechanisms responsible for such alterations are still unknown and some data have been discussed on a speculative basis, there are several findings underlining the importance to further investigate the long-term consequences of a GM-diet and the potential synergistic effects with ageing, xenobiotics and/or stress conditions.

Acknowledgments We thank M. Storaci of the Istituto Zooprofilattico Sperimentale dell'Umbria e delle Marche for his assistance in mouse breeding. This work was supported by the Agenzia Servizi Settore Agroalimentare delle Marche, Italy.

References

Anantharaju A, Feller A, Chedid A (2002) Aging liver. Gerontology 48:343–353

Bernhard W (1969) A new staining procedure for electron microscopical cytology. J Ultrastruct Res 27:250–265

Bjellqvist B, Pasquali C, Ravier F, Sanchez JC, Hochstrasser D (1993) A nonlinear wide-range immobilized pH gradient for two-dimensional electrophoresis and its definition in a relevant pH scale. Electrophoresis 14:1357–1365

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Carreras MC, Poderoso JJ (2007) Mitochondrial nitric oxide in the signaling of cell integrated responses. Am J Physiol Cell Physiol 292:C1569–1580

Dai G, He L, Chou N, Wan YJ (2006) Acetaminophen metabolism does not contribute to gender difference in its hepatotoxicity in mouse. Toxicol Sci 92:33–41

Doull J, Gaylor D, Greim HA, Lovell DP, Lynch B, Munro IC (2007) Report of an Expert Panel on the reanalysis by Séralini of a 90-day study conducted by Monsanto in support of the safety of a genetically modified corn variety (MON 863). Food Chem Toxicol 45:2073–2085

Durante W, Johnson FK, Johnson RA (2007) Arginase: a critical regulator of nitric oxide synthesis and vascular function. Clin Exp Pharmacol Physiol 34:906–911

Ewen SW, Pustzai A (1999) Effects of diets containing genetically modified potatoes expressing *Galanthus nivalis* lecitin on rat small intestine. Lancet 354:1353–1354

Fakan S (2004) Ultrastructural cytochemical analyses of nuclear functional architecture. Eur J Histochem 48:5–14

Frasca D, Nguyen D, Riley RL, Blomberg BB (2003) Decreased E12 and/or E47 transcription factor activity in the bone marrow as well as in the spleen of aged mice. J Immunol 170:719–726

Fujita T (1999) Senescence marker protein-30 (SMP30): structure and biological function. Biochem Biophys Res Commun 254:1–4

Fukaya Y, Yamaguchi M (2004) Regucalcin increases superoxide dismutase activity in rat liver cytosol. Biol Pharm Bull 27:1444–1446

Galy V, Olivo-Marin JC, Scherthan H, Doye V, Rascalou N, Nehrbass U (2000) Nuclear pore complexes in the organization of silent telomeric chromatin. Nature 403:108–112

Gharahdaghi F, Weinberg CR, Meagher DA, Imai BS, Mische SM (1999) Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: a method for the removal of silver ions to enhance sensitivity. Electrophoresis 20:601–605

Gilca M, Stoian I, Atanasiu V, Virgolici B (2007) The oxidative hypothesis of senescence. J Postgrad Med 53:207–213

Granby K, Johannesen S, Vahl M (2003) Analysis of glyphosate residues in cereals using liquid chromatography–mass spectrometry. Food Addit Contam 20:692–698

Hallen A (2002) Accumulation of insoluble protein and ageing. Biogerontology 3:307–315

Hildebrand R (1980) Nuclear volume and cellular metabolism. Adv Anat Embryol Cell Biol 60:1–54

Hochstrasser DF, Patchornik A, Merril CR (1988) Development of polyacrylamide gels that improve the separation of proteins and their detection by silver staining. Anal Biochem 173:412–423

Honore B, Ostergaard M, Vorum H (2004) Functional genomics studied by proteomics. Bioessays 26:901–915

Jameson CW (2004) Towards a unified and interdisciplinary model of ageing. Med Hypotheses 63:83–86

Justo R, Boada J, Frontera M, Oliver J, Bermúdez J, Gianotti M (2005) Gender dimorphism in rat liver mitochondrial oxidative metabolism and biogenesis. Am J Physiol Cell Physiol 289:C372–378

Kato K, Giulivi C (2006) Critical overview of mitochondrial nitric-oxide synthase. Front Biosci 11:2725–2738

Kuiper HA, König A, Kleter GA, Hammes WP, Knudsen I, European Network on Safety Assessment of Genetically Modified Food Crops (ENTRANSFOOD) (2004) Safety assessment, detection and traceability, and societal aspects of genetically modified foods. European Network on Safety Assessment of Genetically Modified Food Crops (ENTRANSFOOD). Food Chem Toxicol 42:1195–1202

Kumar AN, Kalyankar GD (1984) Effect of steroid hormones on age dependent changes in rat arginase isoenzymes. Exp Gerontol 19:191–198

Lafarga M, Berciano MT, Andres MA (1993) Protein-synthesis inhibition induces perichromatin granule accumulation and intranuclear rodlet formation in osmotically stimulated supraoptic neurons. Anat Embryol 187:363–369

Lin G, Cui YY, Liu XQ (2003) Gender differences in microsomal metabolic activation of hepatotoxic clivorine in rat. Chem Res Toxicol 16:768–774

Malatesta M, Bertoni-Freddari C, Fattoretti P, Baldelli B, Fakan S, Gazzanelli G (2004) Aging and vitamin E deficiency are responsible for altered RNA pathways. Ann N Y Acad Sci 1019:379–382

Malatesta M, Bertoni-Freddari C, Fattoretti P, Caporaloni C, Fakan S, Gazzanelli G (2003a) Altered RNA structural constituents in aging and vitamin E deficiency. Mech Ageing Dev 124:175–181

Malatesta M, Biggiogera M, Manuali E, Rocchi MBL, Baldelli B, Gazzanelli G (2003b) Fine structural analyses of pancreatic acinar cell nuclei from mice fed on GM soybean. Eur J Histochem 47:385–388

Malatesta M, Caporaloni C, Gavaudan S, Rocchi MBL, Tiberi C, Gazzanelli G (2002a) Ultrastructural morphometrical and immunocytochemical analyses of hepatocyte nuclei from mice fed on genetically modified soybean. Cell Struct Funct 27:173–180

Malatesta M, Caporaloni C, Rossi L, Battistelli S, Rocchi MBL, Tonucci F, Gazzanelli G (2002b) Ultrastructural analysis of pancreatic acinar cells from mice fed on genetically modified soybean. J Anat 201:409–416

Malatesta M, Mannello F, Sebastiani M, Cardinali A, Marcheggiani F, Renò F, Gazzanelli G (1998) Ultrastructural characterization and biochemical profile of human gross cystic breast disease. Breast Cancer Res Treat 48:211–219

Malatesta M, Tiberi C, Baldelli B, Battistelli S, Manuali E, Biggiogera B (2005) Reversibility of hepatocyte nuclear modifications in mice fed on genetically modified soybean. Eur J Histochem 49:237–242

Marc J, Le Breton M, Cormier P, Morales J, Belle R, Mulner-Lorillon O (2005) A glyphosate-based pesticide impinges on transcription. Toxicol Appl Pharmacol 203:1–8

Molloy MP, Brzezinski EE, Hang J, McDowell MT, VanBogelen RA (2003) Overcoming technical variation and biological variation in quantitative proteomics. Proteomics 3:1912–1919

Morris ME, Lee HJ, Predko LM (2003) Gender differences in the membrane transport of endogenous and exogenous compounds. Pharmacol Rev 55:229–240

Multhoff G (2006) Heat shock proteins in immunity. Handb Exp Pharmacol 172:279–304

Numao N, Masuda H, Sakai Y, Okada Y, Kihara K, Azuma H (2007) Roles of attenuated neuronal nitric-oxide synthase protein expression and accelerated arginase activity in impairing neurogenic relaxation of corpus cavernosum in aged rabbits. BJU Int 99:1495–1499

Padgette SR, Kolacz KH, Delannay X, Re DB, LaVallee BJ, Tinius CN, Rhodes WK, Otero YI, Barry GF, Eichholtz DA, Peschke VM, Nida DL, Taylor NB, Kishore GM (1995) Development, identification and characterization of a glyphosate-tolerant soybean line. Crop Sci 35:1451–1461

Pastorelli R, Carpi D, Campagna R, Airoldi L, Pohjanvirta R, Viluksela M, Hakansson H, Boutros PC, Moffat ID, Okey AB, Fanelli R (2006) Differential expression profiling of the hepatic proteome in a rat model of dioxin resistance: correlation with genomic and transcriptomic analyses. Mol Cell Proteomics 5:882–894

Patel NN, Crincoli CM, Kennedy EL, Frederick DM, Tchao R, Harvison PJ (2008) Effect of gender, dose, and time on 3-(3,5-dichlorophenyl)-2,4-thiazolidinedione (DCPT)-induced hepatotoxicity in Fischer 344 rats. Xenobiotica 38:435–449

Peixoto F (2005) Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. Chemosphere 61:1115–1122

Pujol G, Soderqvist H, Radu A (2002) Age-associated reduction of nuclear protein import in human fibroblast. Biochem Biophys Res Comm 274:354–358

Rea IM, McNerlan S, Pockley AG (2001) Serum heat shock protein and anti-heat shock protein antibody levels in aging. Exp Gerontol 36:341–352

Richard S, Moslemi S, Sipahutar H, Benachour N, Seralini GE (2005) Differential effects of glyphosate and roundup on human placental cells and aromatase. Environ Health Perspect 113:716–720

Roijkind M, Dominguez-Rosales JA, Nieto N, Greenweld P (2002) Role of hydrogen peroxide and oxidative stress in healing responses. Cell Mol Life Sci 59:1872–1891

Sanvido O, Romeis J, Bigler F (2007) Ecological impacts of genetically modified crops: ten years of field research and commercial cultivation. Adv Biochem Eng Biotechnol 107:235–278

Schmitt E, Gehrmann M, Brunet M, Multhoff G, Garrido C (2007) Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. J Leukoc Biol 81:15–27

Schmucker DL (1990) Hepatocyte fine structure during maturation and senescence. J Electron Microsc Tech 14:106–125

Schwarzacher HG, Wachtler F (1993) The nucleolus. Anat Embryol 188:515–536

Séralini GE, Cellier D, Spiroux de Vendomois J (2007) New analysis of a rat feeding study with a genetically modified maize reveals signs of hepatorenal toxicity. Arch Environ Contam Toxicol 52:596–602

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal Chem 68:850–858

Sverko V, Sobocanec S, Balog T, Marotti T (2004) Age and gender differences in antioxidant enzyme activity: potential relationship to liver carcinogenesis in male mice. Biogerontology 5:235–242

Szarek J, Siwicki A, Andrzejewska A, Terech-Majewska E, Banaszkiewicz T (2000) Effects of the herbicide Roundup on the ultrastructural pattern of hepatocytes in carp (*Cyprinus carpio*). Mar Environ Res 50:263–266

Tobisawa M, Tsurusaki Y, Yamaguchi M (2003) Decrease in regucalcin level and enhancement of protein tyrosine phosphatase activity in rat brain microsomes with increasing age. Int J Mol Med 12:577–580

Tong BC, Barbul A (2004) Cellular and physiological effects of arginine. Mini Rev Med Chem 4:823–832

Torres AM (1996) Gender-differential liver plasma membrane affinities in hepatic tetrabromosulfonephthalein (TBS) uptake. Biochem Pharmacol 51:1117–1122

Trabalza-Marinucci M, Brandi G, Rondini C, Avellini L, Giammarini C, Costarelli S, Acuti G, Orlandi C, Filippini G, Chiaradia E, Malatesta M, Crotti S, Antonini C, Amagliani G, Manuali E, Mastrogiacomo AR, Moscati L, Haouet MN, Gaiti A, Magnani M (2008) A three year longitudinal study on the effects of a diet containing genetically modified Bt176 maize on the health status and performance on sheep. Livestock Sci 113:178–190

Tipton KD (2001) Gender differences in protein metabolism. Curr Opin Clin Nutr Metab Care 4:493–498

Tudisco R, Lombardi P, Bovera F, d'Angelo D, Cutrignelli MI, Mastellone V, Terzi V, Avallone L, Infascelli F (2006) Genetically modified soya bean in rabbit feeding: detection of DNA fragments and evaluation of metabolic effects by enzymatic analysis. Anim Sci 82:193–199

Valdez LB, Zaobornyj T, Alvarez S, Bustamante J, Costa LE, Boveris A (2004) Heart mitochondrial nitric oxide synthase. Effects of hypoxia and aging. Mol Aspects Med 25:49–59

Vecchio L, Cisterna B, Malatesta M, Martin TE, Biggiogera M (2004) Ultrastructural analysis of testes from mice fed on genetically modified soybean. Eur J Histochem 48:449–453

Voss MR, Stallone JN, Li M, Cornelussen RN, Knuefermann P, Knowlton AA (2003) Gender differences in the expression of heat shock proteins: the effect of estrogen. Am J Physiol Heart Circ Physiol 285:H687–H692

Wei Y, Chan LL, Wang D, Zhang H, Wang J, Dai J (2008) Proteomic analysis of hepatic protein profiles in rare minnow (*Gobiocypris rarus*) exposed to perfluorooctanoic acid. J Proteome Res 7:1729–1739

Witte MB, Barbul A (2003) Arginine physiology and its implication for wound healing. Wound Repair Regen 11:419–423

Yamaguchi M (2005) Role of regucalcin in maintaining cell homeostasis and function. Int J Mol Med 15:371–389

Yamaguchi M, Tsurusaki Y, Misawa H, Inagaki S, Ma ZJ, Takahashi H (2002) Potential role of regucalcin as a specific biochemical marker of chronic liver injury with carbon tetrachloride administration in rats. Mol Cell Biochem 241:61–67

Zhang HJ, Drake VJ, Morrison JP, Oberley LW, Kregel KC (2002) Selected contribution: differential expression of stress-related genes with aging and hyperthermia. J Appl Physiol 92:1762–1769