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Hepatorenal and genotoxic effects of genetically modified quail meat in a 90-day dietary toxicity study in mice

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Abstract: The aim of this study was to evaluate the possible hepatorenal toxicity and genotoxicity from eating meat meal of genetically modified (GM) Japanese quail in 90 days dietary experiment using micronucleus (MN) test, mitotic index (MI) and RAPD-PCR for genotoxicity assay, ALT, AST, urea and creatinine for hepatorenal toxicity. Four groups of Swiss male mice were used. Control 1 received balanced ration, control 2 received 20% non-GM quail meat, treated 1 received 20% GM quail meat meal, and treated 2 received 40% GM quail meat meal. Minor differences in body weight were observed between the 4 groups. 40% GM quail meat meal induced hepatorenal toxicity; meanwhile 20% induced renal toxicity only. GM quail meat meal induced genotoxicity by increased MN and nuclear buds caused by the 40%, while 20% caused nuclear buds only. Our RAPD fingerprints showed differences between the individual of both controls and both treatments in the number and intensity of the amplified DNA bands. The combined data of MN, nuclear buds and RAPD data indicate the genotoxic effect of both doses of GM quail meat which have nearby effects on fragmentation of genetic material.

Keywords: GM food, genotoxicity, MN, hepatorenal toxicity, RAPD-PCR

Introduction

The World Health Organization (WHO, 2002) defines genetically modified organisms (GMOs) as those organisms in which the genetic material has been altered in a way that does not occur naturally. The technology used allows selected individual genes to be transferred from an organism into another, and also between non-related species, by gene injection into embryo pronucleus or into egg cytoplasm and the embryo becoming transgenic animals (Whitelaw, 2004). It is a well-known fact that as socio-economic status of global communities rise, consumers demand more dietary animal protein as meat and milk. Genetic engineering of livestock and poultry will allow such improvements through production of transgenic livestock which contain an exogenous growth hormone (GH) gene which associated with increased growth rate (Seidel, 1999; Hew *et al*., 1995; Devlin *et al*., 1995).

Recognizing the rising concern among the world population about the safety and nutritional aspects of foods derived from biotechnology. The safety assessment of GM derived food follows a comparative approach between GM with their non-GM counterparts in order to identify unexpected differences which subsequently are assessed with respect to their potential impact on the environment, safety for humans and animals (Bakshi, 2003; Brake and Evenson, 2004). Extensive experience has been built up in recent decades from the safety and nutritional testing in animals on whole GM food and feed (Momma *et al*., 2000; El-Sanhoty *et al.*, 2004). These studies reported damaged immune system, digestive problems, smaller internal organs, allergy, decreased body weight and hepatorenal toxicity (Vidal, 2002; Domingo, 2007; Seralini *et al*., 2007).

Genetic modification (GM) of food animals has been achieved since the early 1980s. However, there are many opinions but very scarce data on the potential health risks of GM foods animals or birds. Cummins and Ho (2006) suggested that foods derived from genetically modified animals are far from safe. They are likely to be contaminated by potent vaccines, immune regulators, and growth hormones, as well as nucleic acids, viruses, and bacteria that have the potential to create pathogens and to trigger cancer.

The present study was carried out to assesses the possible genotoxicity indicated by mitotic index (MI), micronucleus assay (MN) and DNA fragmentation using Random Amplified Polymorphic DNA (RAPD), renal and hepatic toxicity from eating meat meal of Japanese quails produced by introducing broiler growth hormone gene into the embryonic cell

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of Japanese quails.

Materials and Methods

Animals

Male white Swiss mice aged 10–12 weeks were used. The animals were obtained from a closed random bred colony at Faculty of Veterinary Medicine, Alexandria University, Egypt. The mice were maintained on food and water ad libitum and housed in four groups of five in micro isolator cages. The animals were acclimatized for 1 week prior to usage. The Local Ethics Committee approved the study.

Body weight

All animals were individually weighted once a week for 12 wks. The animals were sacrificed 6 days following the last weight to complete 90 days daitery exposure.

Processing of quails muscles

GM quails modified by chicken growth hormone gene and non-GM quails were obtained from Department of Animal and Fish Production, Faculty of Agriculture-Saba Basha, Alexandria University as a research product done by Mohamed (2009). GM and non-GM quails were cooked, their muscles were removed and crushed by a food processor, completely dried in hot oven and were ground by food processor into fine meat meal powder, which added on mice ration.

Experimental design

All groups received their diets for 90 days; the groups of animal were as following:
Group1 (Control 1- C1) was given nutritionally balanced diet. Group 2 (Control 2 - C2) received nutritionally balanced diet with 20% non-gentically modified quail meat meal. Group 3 (Treated 1-T1) received balanced diet with 20% genetically modified quail meat meal. Group 4 (Treated 2-T2) received balanced diet with 40% genetically modified quail meat meal.

Kidney and Liver function tests

Animals were anaesthetized with ether, and then blood was withdrawn from the inner canthus of the eye (Waynforth, 1980). The blood was centrifuged with 3000 rpm for 10 min. to separate the serum and stored frozen at -20^{o}C until assayed for alanine aminotransferase (ALT) and aspartate aminotransferase (AST) according to Reitman and Frankel (1957), urea (Patton and Crouch, 1977) and creatinine (Henry, 1975).

Genotoxicity studies

Cytogenetic analysis

Mitotic Index (MI)

The mice were sacrificed 1–2 hr after injection of 4 mg/kg b.wt. colchicine.Bone marrow preparation from one femur were made according to Giri *et al* (1986). The cells were spread into clean slide, air dried stained with Gur Giemsa, the mitotic indices (MI) were calculated from 1000 cells per animals.

Micronucleus (MN) test.

MN was prepared from the other femur according to Schmid (1976), 500 polychromatic erythrocytes were demonstrated for each animal.

DNA and RAPD-PCR

DNA Extraction

The DNA was isolated from liver of three mice of each group, a sample weight (20 – 50 mg) of liver was then extracted by phenol/chloroform extraction method followed by ethanol precipitation (Sambrook *et al*., 2001).

RAPD- PCR

RAPD PCR was performed using the primers designated 111, 114, 115, 127, 134, 137, OPA2, OPA4, OPA16 and OPA17. The nucleotide sequences of these primers and G-C content and annealing temperature are listed in Table (1). The calculated melting temperatures (Tm) of each primer were estimated as follows: Tm = 4(G + C) + 2 (A + T). RAPD-PCR was carried out in 25 µl reaction volume containing 2 µl test DNA sample (5 ng/µL), 100 pmol of RAPD primers, 3.5 mM $MgCl_2$, 2.5 µl 10X PCR Gold Buffer, 200 mM of each deoxynucleotide triphosphate (dNTP) and 0.4 U Taq Polymerase (Promega) (Savva, 2000).

The cycling profile was: 95^{o}C for 5 min in precycle, followed by 35 cycles of 95^{o}C denaturation for 1 min, primer annealing were shown in Table (1) and extension at 72^{o}C for 1 min. Final primer extension continued for an additional 10 min to allow the complete elongation of all amplifications. PCR product of each sample (8 µl) was mixed with 2 µl loading buffer, and analyzed by electrophoresis in 2% agarose gels stained with ethidium bromide. The PCR products were identified by size using a 100 bp ladder.

Scoring, data analysis of RAPD and Dendogram construction (phylogenic tree)

The DNA bands were scored for their presence

as (1) or absence as (0) in the RAPD profile of 12 mice belonging to 4 groups. Comparative data were generated between the 12 variable. Distance matrix data were used to construct a dendrogram using the unweighted pair-group with arithmetic mean (UPGMA). The cluster analysis and dendrogram construction was performed with Statistica 5 (1995).

Statistical analysis

Data were submitted to analysis of variance (ANOVA), P≤0.05, to compare the treated groups with the control group. When significant difference was found, means were compared used Duncan's multiple range test (Duncan, 1955). Calculations were done using SAS system (SAS, 1987).

Results

Body weight

Weekly body weight in 90 day dietary toxicity in all groups showed normal growth curve, however, no significant differences between all groups were observed at weeks 1, 3 and 5. During the other weeks, each individual group, either the control (1 and 2) or treated (20%, 40% GM quail meat meal) showed a significant increase in body weight at different time (Figure 1). Control 2 has higher body weight at 2nd week, T1 group (20% GM quail meat) has higher body weight at 6th week. T2 group (40% GM quail) has higher body weight at 9th week.

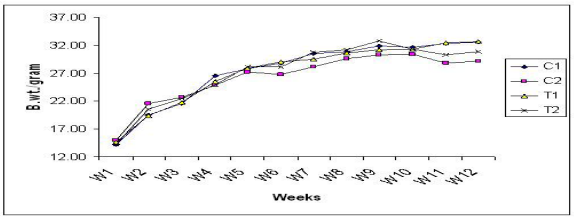

Figure 1. Mean weekly body weight in 4 groups, control 1, control 2, treated 1 and treated 2 in 90 day's deiaty experiment of 20% and 40% GM quail meat

Liver and Kidney function

Concerning the biochemical parameters of kidney and liver functions. It was observed that serum urea, creatinine, ALT and AST levels were significantly ($p < 0.05$) increased in mice received 40% genetically modified quail meat in comparison with those taken only a balanced ration and /or non genetically modified quail meat (Table 2). Meanwhile, those received 20 % genetically modified quail meat showed a significant elevation in serum creatinine but the other parameters were slightly (P>0.05) elevated in comparison with other groups that received non-GM quail meat (Table 2). Additionally, these parameters were not significantly differed between the groups that taken either 20 or 40 % quail meat (Table 2).

Table 2. Liver and kidney function parameters in 90-day dietary toxicity study in mice fed 20% and 40% of GMO quail meat meal

AST	ALT	Creatinin	Urea	Groups
42.75±1.11^{b}	23.25±2.66^{b,c}	0.23±0.03^{b}	22.0 ±1.08^{b}	C 1
40.75±3.25^{b}	19.25±1.11^{c}	0.21±0.01^{b}	23.75±1.03^{b}	C2
47.50±3.20^{a,b}	27.50±1.55^{a,b}	0.35±0.07^{a}	25.75±0.02^{a,b}	T1
55.75±2.59^{a}	31.00±3.11^{a}	0.40±0.06^{a}	29.25±3.04^{a}	T2

Each value represents the mean±S.D.of five animals.
Values with different letters at the same column are significantly different at P≤0.05 (ANOVA with Duncan's multiple range test)
C1: Control 1 C1: Control 2 T1: Treated 1 T1: Treated 2

Cytogenetic analysis

It was observed that ingestion of 20% and 40% GM quail meat caused a significant increase in nuclear buds compared to both control. Furthermore, 20% GM quail meat caused elevation of the rate of the MN and 40 % GM quail meat caused slight elevation of MN compared to the control. No significant differences were observed in the rate of MN between 20% and 40% GM quail meat or between mice received balanced ration and those received 20% of non-GM quail meat. Feeding mice with 20% and 40% GM quail meat significantly increased mitotic index compared with both control. The group received 20% GM quail meat has higher mitotic index than group received 40% (Table 3). Furthermore, significant differences were also observed between the C1 (received balanced ration.) and C2 (20 % non- GM quail meat) (Table 3).

Table 3. Micronuclus (MN), nuclear budding and mitotic index (MI) in a 90-day dietary toxicity study in mice fed 20% and 40% of GMO quail meat meal

MI	Nuclear budding	MN	Groups
18.20 ± 1.39^{d}	2.20 ± 0.58^{b}	1.60 ± 0.68^{b}	C 1
28.20 ± 4.26^{c}	3.80 ± 0.73^{b}	1.60 ± 0.40^{b}	C2
72.60 ± 4.82^{a}	6.00 ± 1.23^{a}	3.40 ± 0.93^{a}	T1
44.80 ± 10.73^{b}	6.20 ± 2.16^{a}	2.80 ± 0.92^{a,b}	T2

Each value represents the mean±S.D. of five animals.
Values with different letters at the same column are significantly different at P≤0.05 (ANOVA with Duncan's multiple range test)
C1: Control 1 C1: Control 2 T1: Treated 1 T1: Treated 2

RAPD- PCR

In initial experiment a total ten RAPD primers were screened on genomic DNA of all treated and untreated groups, two primers gave no bands, three primers gave smear, two primers gave one band, only three primers gave visible and reproducible bands as shown in Table 1. These primers were 115, 137, OPA4 revealed polymorphic and unique bands as shown in Figure 2. They produced 31 bands ranged from 50-1500 bp. The RAPD fingerprints showed differences between the individual of C1 and C2 in the number and intensity of the of the amplified DNA bands. Concerning to T1 (20% GM quail) and T2 (40% GM quail), they have change in the intensity and number of the DNA bands compared to the both control. Little differences were observed in the banding pattern between the individuals in the group

Table 1. PCR primers used in RAPD-PCR, G-C content and annealing temperature

Primer name	Primer sequence	G-C%	Annealing temp.	PCR product
115	TTCCGC GGG C	80	42	good
137	GGT CTC TCCC	70	40	good
OPA4	AATCGGGCTG	60	43	good
111	AGTAGA CGGG	60	35	Smear
114	TGACCG AGAC	60	35	Smear
127	ATCTGG CAGC	60	34	Smear
134	AAC ACACGAG	50	35	One band
OPA2	TCGGCGATAG	60	35	No bands
OPA16	AGCCAGCGAA	60	40	One band
OPA 17	GACCGCTTGT	60	42	No bands

receive 20%GM quail and those received 40% GM quail meat. They have the same banding pattern in primer 115 and many sharing bands in primers 137 and OPA4. The results of RAPD profile of primer 115 and OPA4 in 12 variables including both control and treated groups are illustrated in Figure 2.

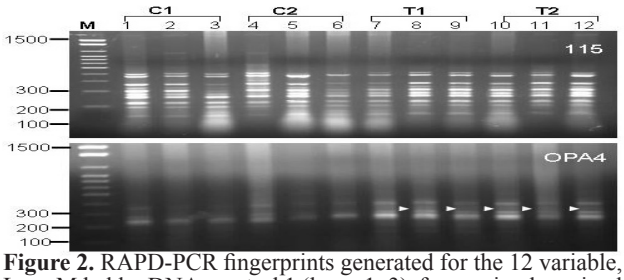

Figure 2. RAPD-PCR fingerprints generated for the 12 variable, Lane M ladder DNA. control 1 (lanes 1–3) from animal received balanced ration, control 2 (lanes 4–6) received 20% non-GM quail meat, treated 1 (lanes 7-9) received 20% GM quail meat, and treated 2 (lanes 10-12) received 40% GM quail meat

Dendrogram of Figure 3 is a possible graphical representation of RAPD data obtained. In our analysis the dendrogram showed the relationship between treated animals in both groups (20%, 40% GM quail meat) in comparison with those in both control (C1, C2). Different polymorphic profile obtained in different groups in RAPD-PCR were also observed in dendrogram clustering. Variations within the individual of each group were also observed (Figure 3). Clustering of the treated groups (T1, T2) indicate their similarity compared with both control groups.

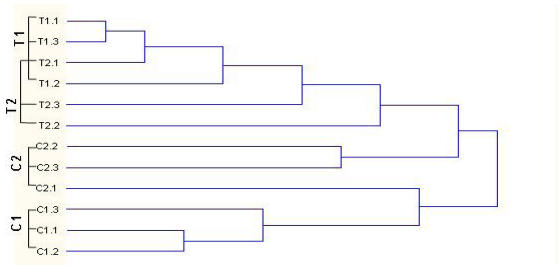

Figure 3. UPGMA dendrogram showing graphical representation of RAPD data of 12 variable, control 1 received balanced ration, control 2 received 20% non-GM quail meat, treated 1 received 20% GM quail meat, and treated 2 received 40% GM quail meat

Discussion

A safety evaluation concept has been developed for GM organisms and crops, which utilize a systematic approach. Importantly, there is an urgent need to develop comprehensive toxicological/nutritional methods to screen for the unintended potentially deleterious consequences for human/animal health of genetic manipulation to pinpoint the problems of the incorporation of the GM foodstuff into the food chain (Ewen and Pusztai, 1999). Toxicity tests in laboratory animals play an important role in ensuring the safety of chemicals, drugs and food components (FAO/WHO, 2001; OECD, 2001).

In this study we evaluated the possible hepatorenal and genotoxicity from eating meat meal from quails genetically modified with chicken growth hormone gene. Quail meat used in our study exposed to heat during its processing. Smith *et al*. (2000) found that DNA is not degraded under most commercial feed processing conditions. Ewen and Pusztai (1999b) demonstrated that no significant differences were observed in rat from eating raw or boiled GM potato in inducing hyperplastic growth of stomach and intestinal mucosa. Chan *et al*. (1998) found that milk and dairy products from cows treated with the genetically engineered bovine growth hormone (bGH) milk contain an increased amount of the hormone IGF-1, which is one of the highest risk factors associated with breast and prostate cancer.

Our results indicate that all groups have normal growth curve and no significant differences were observed in body weights between the control and the treated groups in most weeks. Except for few individual weeks, each group showed marked increase in the body weights over the other groups. Chainark *et al.* (2006) demonstrated no marked differences in growth in fish fed different levels of GM or non-MG diet. Similar results were also obtained in feeding studies of modified and unmodified feed has been reported for other animals, like swine, poultry and dairy cattle (Donkin *et al*., 2000; Cromwell *et al*., 2002; Rossi *et al*., 2005).

Significant variations were observed in mitotic index between all groups in this study. Control 2 received 20% non-GM quail meat has higher MI than control 1 which received nutritionally balanced diet. The highest MI was observed in T1 group which received 20% GM quail meat. Goodlad (1981) observed that MI increased rapidly when the diet of sheep contain higher protein, then declined after several days to new level which slightly higher than observed initially. He also observed that the duration of cell cycle using labeled mitosis curve was decreased

when the cell proliferation was stimulated by higher protein content of the diet and this decrease in cell cycle time was mainly the result of the decreased duration of the phase of DNA synthesis (S phase). We suggested that 20% GM quail meat has the highest effect on reducing the cell cycle duration which reflected by the highest level of MI.

Ingestion of 40% of GM quail meat meal induced liver and kidney toxicity indicated by increased serum urea and creatnine, ALT and AST. Meanwhile, 20% GM quail elevate only serum creatinine. Seralini *et al*. (2007); de-Vendômois *et al*. (2009) have used 90 days dietary experiment to assess the sub chronic effects of GMO. Several convergent factors appear to indicate liver and kidney problems as end points of GMO diet effects. Tudisco *et al*. (2006) found that rabbits fed GM soy showed enzyme function disturbances in kidney and heart. Sakamoto *et al.* (2007) demonstrated several differences in serum biochemical parameters and histological findings between the rats fed the GM soybeans. Séralini *et al*. (2007) compared between GM corn -treated rats and the controls fed with an equivalent normal diet in 90-days dietary experiment which showed dose-related significant variations hepatorenal toxicity. GMOs new side effects linked with GM maize consumption, which were dose-dependent effects were also noticed in the heart, adrenal glands, spleen and haematopoietic system (de Vendômois, 2009).

Micronuclei (MN) and other nuclear anomalies such as nuclear buds are biomarkers of genotoxic events and chromosomal instability. MN can originate during anaphase from lagging acentric chromosome or chromatid fragments caused by DNA strand breaks, misrepair of DNA breaks or unrepaired DNA breaks (Attia *et al*., 2009; Fenech *et al*., 2011). Animal received 40% of GM quail meat has a higher MN and nuclear buds compared with both control. Animals which received 20% GM quail meat food has increased nuclear budding compared with the control. We suggested that GM quail meat has genotoxic effect and no significant differences were observed in the number of MN or nuclear buds between the groups received 20% and 40% GM quail meat.

RAPD is a reliable, sensitive and reproducible and has the potential to detect a wide range of DNA damage (e.g. DNA adducts, DNA breakage) as well as mutations (point mutations and large rearrangements), therefore can be applied to genotoxicity studies (Atienzar *et al.*, 1999; Atienzar and Jha, 2006). Our RAPD fingerprints showed differences between the individual of both controls and both treatments in the number and intensity of the of the amplified DNA bands. Changes observed in the DNA profiles such as modifications in band intensity and loss of bands may be due to the changes in oligonucleotide priming sites mainly due to genomic rearrangements and less likely to point mutations or DNA damage in the primer binding sites (Nelson *et al*., 1996). Dendrogram clustering of RAPD data indicate the differences between all groups and the clustering of the individual in the groups treated with 20% and 40% GM quail meat indicate their similarity. The combined data of MN, nuclear budding and RAPD data indicate the genotoxic effect of both doses of GM quail meat which have nearby effect on fragmentation of genetic material.

Food derived from genetically modified animals pose several kinds of health risks. While scientists originally assumed that the inserted genes would only add a particular desired trait to the organism, new evidence suggests that the host's normal natural genes can gets switched off, turned on permanently, damaged, or altered in the process and that's just some of the many ways that GM foods may create unpredicted and potentially dangerous side effects (Ho and Steinbrecher, 1998; Brigulla and Wackernagel, 2010). We conclude that GM quail meat induced hepatorenal toxicity which is dose dependent. Meanwhile, the higher and lower doses of GM quail meat induced genotoxic effect.

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