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Mass spectrometric evidence for the modification of small molecules in a cobalt-60 irradiated rodent diet[&]

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Abstract

The purpose of this study was to investigate the effect of radiation on the content of animal diet constituents using global metabolomics. Aqueous methanolic extracts of control and cobalt-60 irradiated NIH 7001 diets were comprehensively analyzed using nanoLC-MS/MS. Among the over two thousand ions revealed by XCMS followed by data preprocessing, 94 positive and 143 negative metabolite ions had greater than 1.5 fold changes and p-values <0.01. Use of Metaboanalyst statistical software demonstrated complete separation of the irradiated and non-irradiated diets in unsupervised principal components analysis and supervised partial least squares discriminant analysis. Irradiation led to an increase in the content of phytochemicals such as glucosinolates and oxidized lipids in the diet. Twenty-eight metabolites that were significantly changed in the irradiated samples were putatively identified at the level of molecular formulae by MS/MS. MS/MS^{ALL} analysis of chloroform-methanol extracts of the irradiated diet showed increased levels of a number of unique linoleic acid-derived branched fatty acid esters of hydroxy fatty acids. These data imply that Gamma-irradiation of animal diets causes chemical changes to dietary components which in turn may influence the risk of mammary cancer.

Keywords

animal diet; Cobalt-60 irradiation; mass spectrometry; oxidized lipids; metabolomics

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Conflict of interest

The authors declare that there are no conflicts of interest.

Introduction

The importance of variability in the diet in animal models of disease is well appreciated and is an important consideration regarding the reproducibility of research funded by NIH. It's been 40 years since the American Institute of Nutrition (AIN) developed criteria for diets used in experimental research. This involved a shift from an undefined laboratory chow diet consisting of mixtures of wheat, fishmeal and soy proteins to defined diets based on casein, oils and vitamin mixtures. The first of these, the AIN76A diet was developed in the late 1970s (1) and was reformulated in the 1990s as AIN93G and AIN93M (2), optimized for growth and maintenance, respectively. Despite this improvement in the nutrition of the rodents, it nonetheless also affected on the biological outcomes of experiments. When the transition from the lab chow diet to AIN76A diet occurred, the number of mammary tumors induced by N-methyl-N-nitrosourea (MNU) in female Sprague-Dawley rats rose sharply without any changes in growth rate and final body weight (3). This suggested that a component of the lab chow diet had chemopreventive properties and led to close examination of the role of soy and its isoflavones in breast cancer prevention (3). However, surprisingly, exposure of rats to the isoflavone genistein, once they were established on AIN-76A diet and had reached 40 days of age, had no effect on numbers of mammary tumors induced by MNU (4,5). This suggested that there were (early) windows of exposure that were relevant to diet-induced mammary cancer prevention.

In related studies, attempts by the NCI chemoprevention program in the 1990s to test combinations of formerly effective chemopreventive agents failed. Examination of experimental records revealed that these previous chemopreventive effects were obtained using a laboratory chow diet. Accordingly, the NCI chemoprevention program reverted to use of laboratory chow diet (containing soy, wheat and fishmeal). Importantly, the chemopreventive effect of soy isoflavones given in the young adult period of life became evident again when they were added to laboratory chow diet (5). This suggested that exposure to soy prior to and after weaning is critical to enable isoflavones to prevent formation of mammary tumors. It confirmed previous observation that addition of genistein alone to the semi-purified AIN76A diet in early life is chemopreventive (4).

As an appreciation of the role of the microbiome in biomedical research models has evolved (plus a separate concern about the stability of stored diets), commercial diet manufacturers have introduced Gamma-irradiation of diets with cobalt-60 to reduce microbial contamination. Many institutional animal resource programs have changed to using these irradiated diets. One of us (CJG) using these irradiated diets in the studies of breast cancer chemoprevention in rats, noted that the number of mammary tumors were 40% less than when using non-irradiated diets. Since there was a risk that the irradiated and non-irradiated diets were not strictly comparable since they had come from different batches of diets over several years, a single batch of NIH 7001 diet was prepared by Envigo and divided into two parts, one not receiving radiation (0 kGy) and the other exposed to 20–50 kGy of cobalt-60 Gamma-radiation. This experiment confirmed the earlier result in that there were 38% less tumors in animals consuming the irradiated diet (C. J. Grubbs, unpublished results).

The goal of this survey study, therefore, was to determine the effects of γ -radiation on the small molecule metabolome of the diet using a global LC-mass spectrometry approach. Compounds detected on the basis of their LC retention times and precursor ion masses were subjected to univariate and multivariate statistical analysis. In addition, the hydrophobic lipid components of the diet were examined using a comprehensive MSMS^{ALL} approach. Ions shown to be significantly changed by the γ -irradiation were analyzed by inspection of their accurate mass MS/MS spectra to obtain preliminary identification.

Materials and methods

Reagents

Solvents (acetonitrile, chloroform and methanol) were the best grades available. The lipid internal standard, C17 ceramide, was purchased from Avanti, Alabaster, AL.

Diets

Envigo (Indianapolis, IN) prepared a batch of NIH 7001 diet (composition provided in supplemental Table 1). Both non-irradiated (control) and irradiated diets were transferred to UAB for use in the chemoprevention experiments. The diets were stored at 4°C during the feeding experiments. During this time, aliquots for biochemical analysis were removed and stored at -20°C under N₂.

Diet processing for metabolomics analysis

LC-MS analysis

Ground, pelleted diet samples (1 g) were extracted in triplicate with ice-cold methanol-water (4:1, by volume) by shaking for 2 h under N₂. The mixtures were centrifuged at 3,000 × g for 10 min at 4°C. The supernatants were carefully decanted and taken to dryness under N₂. The dried extracts were re-suspended in 1 ml double-distilled H₂O and centrifuged at 14,000 × g for 10 min at 4°C.

Total Lipid analysis

Lipid extraction of the diets was carried out using the Bligh and Dyer method (6). Briefly, each ground diet sample (6 mg) was homogenized in water (1 mL). Methanol (2.5 ml), chloroform (1.25 ml) and internal standard (C₁₇ ceramide, 0.1 µg/mL, 10 µl) were added to the homogenate and sonicated for 10 s. Then an additional 1.0 ml water and 1.25 ml chloroform were added and samples shaken vigorously. Each sample was centrifuged and the lower chloroform layer was removed carefully. The upper layer was re-extracted following the same procedure. The chloroform-soluble fraction was evaporated to dryness under N₂ for analysis.

Metabolomics analysis

A clear aqueous solution of diet extract (5 µl) was injected onto an Eksigent reverse-phase C₁₈ pre-column cartridge (0.5 cm × 200 µm ID) in 0.1% formic acid. Metabolites bound to the cartridge were eluted with a 20 min linear gradient of 0–80% acetonitrile in 0.1% formic acid onto an Eksigent ChipLC C₁₈ column (15 cm × 200 µm ID) at a flow rate of 1 µl/min.

The ChipLC column was placed in an Eksigent Nanoflex (SCIEX, Concord, ON, Canada) operating at 45°C. At the end of each analytical run, the column was washed with acetonitrile:0.1% formic acid for 1 min followed by re-equilibration with 0.1% formic acid for 4 min. Eluate from the ChipLC column was passed into the nanoelectrospray ionization interface of a Triple TOF™ 5600 Mass Spectrometer System operating in the negative and positive ion modes. The collision energy was set to 35 V, curtain gas to 20, GS1 and GS2 to 15, spray voltage to 5500 V (positive ion mode)/4500 V (negative ion mode), and temperature to 400°C. In each duty cycle lasting 1.25 s, high mass resolution MS spectra were collected for 250 ms followed by 50 ms MS/MS spectra of the top 20 most intense molecular ions. Ions producing successful MS/MS spectra were put onto an exclusion list for the next 90 s.

MS/MS^{ALL} analysis

Dried chloroform-methanol extracts were reconstituted with methanol:chloroform (2:1 v/v) with 5 mM ammonium acetate and directly infused at 5 µl/min into a TurboIon electrospray ionization interface and analyzed to record MS and MS/MS positive and negative ion spectra on a Triple TOF™ 5600 System. During MS/MS^{ALL} acquisition, precursor ion isolation windows of 1.2 Da width selected in Q1 are fragmented in the Q2 collision cell and the generated product ions are monitored at high mass resolution by the TOF analyzer. The MS/MS^{ALL} data included a 250 ms survey scan of TOF-MS data from m/z 200–1200, followed by collection of 1000 MS/MS spectra of the selected mass windows (7). The collision energy was set to 35 V, curtain gas to 20, GS1 and GS2 to 15, spray voltage to 5500 V (positive ion mode)/4500 V (negative ion mode), and temperature to 400°C. The total time to carry out the entire experiment was ~8 min.

Data analysis

Collected MS data were inspected using Mzmine 2.21 (8). Putative identities were obtained by submitting the ions to MetaboSearch (<http://omics.georgetown.edu/metaboSearch.html>). Raw MS data were uploaded as .wiff files to XCMSOnline (<https://xcmsonline.scripps.edu>) in order to align metabolite ions across all of the diet extracts. A new feature of XCMSOnline is Activity Network (Connections), based on the software program mummichog, which provides information on the pathways involved as well as (non-pathway) chemical groupings and calculates their statistical significance. XCMS processed data were downloaded from XCMSOnline in .zip format. Once expanded, the data in the Excel report file were filtered to remove ions eluting before 5 min (not bound to the column) or after 25 min (only eluted by the high acetonitrile wash step at the end of the gradient) and ions with integrated peak areas less than 5,000. The data were divided into individual Excel .csv files for each sample containing the median m/z values, median retention times and peak areas, grouped into two folders (irradiated and non-irradiated samples) and uploaded as a .zip file to MetaboAnalyst 3.0 (<http://www.metaboanalyst.ca/MetaboAnalyst/>). The data were normalized using individual sample total ion currents, and subjected to mean centering and Pareto scaling. Accordingly, univariate (Volcano plots) and multivariate (Principal Components Analysis, PCA, and Partial Least Squares Discriminant Analysis, PLSDA) analysis of the data were carried out. For the latter, the metabolites

contributing to the largest separation between the two groups were identified in principal component 1.

Results

Radiation-induced metabolomics changes in the diet

Using XCMSOnline, a total of 317 features were observed in LC-MS data collected in the negative ion mode and 136 features in the positive ion mode that had undergone significant change ($p < 0.05$ and fold change > 1.5) between the irradiated and non-irradiated diets. After cleaning up the downloaded LC-MS data from XCMSOnline to remove early (non-bound) metabolite ions and ions collected in the solvent wash period, a Volcano plot (Fig. 1) revealed 143 negatively charged ions and 94 positively charged ions with fold changes greater 1.5 and p -values < 0.01 . Principal Components Analysis (PCA) and Partial Least Square-Discriminant Analysis (PLS-DA) were performed to assess chemometric separation among the non-irradiated and irradiated diet samples. Metaboanalyst showed complete separation of the irradiated and non-irradiated diets in PCA analyses (Fig. 2A and B).

The molecular formulae of twenty-eight metabolites observed by nanoLC-MS that were significantly changed (mostly up-regulated in the irradiated samples) were determined based on their measured accurate mass and MS/MS product ion interpretation (Table 1).

Effect of radiation on phytochemicals

Aqueous methanolic extracts of irradiated diet showed a series of peaks at R_t 8–16 min in nanoLC-MS analysis that corresponded to glucosinolates. Compared to the control diet, contents of these metabolites were significantly increased in the irradiated samples (Table 1).

The highest increase (13-fold) was noted with deprotonated molecular ion m/z 424.038 (R_t 8.07 min) with product ions m/z 96.961, 195.033, 259.014, 274.989 and 290.997 in its MS/MS spectra (Supplemental Fig. 1A). These product ions are highly specific for glucosinolates and are derived from the cleavage on either side of the thioether bond (9). The presence in the high-resolution MS/MS spectrum of m/z 424.038 also showed a neutral loss of 79.961 Da (SO_3) and an intense product ion m/z 96.961 $[\text{HSO}_4]^-$, indicating the presence of a sulfate group. The exact mass for this ion corresponded to the elemental composition $\text{C}_{14}\text{H}_{19}\text{NO}_{10}\text{S}_2$ (calc. m/z 424.038) and is tentatively identified as a benzyl glucosinolate, most likely to be sinalbin, based on comparison of its MS/MS product ions with those of reference (9).

Similarly, three other compounds m/z 508.100, 522.112, and 536.130 were identified as methylsulfonyl alkyl chain containing glucosinolates on the basis of their high resolution MS/MS spectra. The MS/MS spectra of all these ions showed diagnostic product ions (m/z 290.981, 259.014, 96.961) of glucosinolates (10) (Supplemental Fig. 1B–D). The ion m/z 522.112, possibly one of the isomers of methylsulfonyl nonylglucosinolate, showed 6-fold increase ($p = 0.001$) in peak area in the irradiated sample, compared to control sample (Table 1). These results indicate that ionizing radiation increases the content of glucosinolates in the diet possibly by making them more available since the diet was pelleted and these

compounds may have been encapsulated in non-digested solid particles. This is analogous to differences in bioavailability observed for drugs when prepared in different formulations.

A relatively polar metabolite ion m/z 299.080 (Rt 10.75 min) in negative ion mode had a prominent product ion m/z 137.025 due to the loss of 162.055 (characteristic of a hexose sugar) which on subsequent loss of 43.989 (CO_2) yielded the product ion m/z 93.036. These data are in agreement with an elemental composition for this compound as $\text{C}_{13}\text{H}_{16}\text{O}_8$ (calc. m/z 299.077), potentially a glycoside of salicylic acid. Another glycoside of phenolic acid m/z 357.088 $[\text{M}-\text{H}]^-$ was observed to be up-regulated in the irradiated diet. This precursor ion yielded two intense product ions m/z 153.019 and m/z 109.030 due to subsequent losses of 204.069 and 43.989 Da. The loss of 204.069 may correspond to acetyl hexose sugar. The presence of dihydroxybenzoic acid moiety can be rationalized by the product ion m/z 153.019 (calc. m/z 153.019). These data suggested this compound is an acetyl hexoside of dihydroxybenzoic acid with molecular formula $\text{C}_{15}\text{H}_{18}\text{O}_{10}$ (calc. m/z 357.082).

A phenolic compound m/z 545.130 (Rt 14.29 min) in the positive ion mode showed a neutral loss of 248.053 Da generating the intense product ion m/z 297.077 in its MS/MS spectrum (Supplemental Fig. 2). The neutral loss of 248.053 Da indicated the presence of a malonyl hexosyl residue conjugated with a phenolic compound (11) with elemental composition $\text{C}_{17}\text{H}_{12}\text{O}_5$ (calc. m/z 297.076). Further loss of 42.011 Da (presumably an acetyl group) from the product ion m/z 297.077 produced m/z 255.066. These data suggested this compound is a malonyl conjugate of a polyphenol with molecular formula $\text{C}_{26}\text{H}_{24}\text{O}_{13}$ (calc. m/z 545.129). The content of this compound was significantly higher in the irradiated samples compared to the control diet.

Another ion m/z 591.136 $[\text{M}-\text{H}]^-$ significantly increased in the irradiated samples compared to the control samples. It showed characteristic neutral losses of 144.042, 102.032 and 62.000 Da indicating the presence of a hydroxyl methyl-glutaryl (HMG) moiety (12). The neutral loss of 144.042 Da provided an abundant product ion m/z 447.093 which on further loss of a hexose sugar unit (162.052 Da) gave rise to the product ion m/z 285.042 (Fig. 3). Based on these data, the ion m/z 591.136 was tentatively identified as a flavonoid glycoside-HMG-conjugate with elemental formula $\text{C}_{27}\text{H}_{28}\text{O}_{15}$ (calc. m/z 591.1350).

A relatively polar metabolite (Rt 7.24 min) with m/z 147.029 showed product ions m/z 129.020, 103.040 due to losses of H_2O and CO_2 in its MS/MS spectrum. Neutral losses of CH_3COOH and CO_2 plus H_2O resulted in the prominent product ions m/z 87.011 and 85.031, respectively. These data indicated this compound is most likely arabinoside-1,4-lactone with molecular formula $\text{C}_5\text{H}_8\text{O}_5$ (calc. m/z 147.029).

Lipid oxidation

Table 1 contains a number of oxidized lipids identified by in nanoLC-MS that were significantly altered by the irradiation. Enhancement of metabolite ion m/z 129.054 was observed in the irradiated diet, compared to the control diet (Table 1). Although precise identity of this ion remains unknown, loss of $\text{C}_2\text{H}_4\text{O}$ (44.06 Da) from the precursor ion m/z 129.054 gave rise to m/z 84.985. These MS/MS data together with deprotonated molecular

high resolution mass suggested this compound is probably α -ketoisocaproate ($C_6H_{10}O_3$; calc. m/z 129.056), arising from branched chain amino acids leucine or isoleucine oxidation.

A number of medium chain length oxidized fatty acids were detected with increased ion intensities in the irradiated samples. For example, isobaric ions m/z 159.067 (Rt 11.95 min) and 159.103 (Rt 14.27 min) had product ions due to neutral losses of H_2O , CO_2 and $HCOOH$ (Supplemental Fig. 3A and B). Thus, these compounds were putatively identified as pimelic acid with molecular formula $C_7H_{12}O_4$ (calc. m/z 159.0657) and $C_8H_{16}O_3$ (calc. m/z 159.1021).

An ion m/z 169.087 (with Rt 20.8 min) had losses of CO_2 and $HCOOH$ producing intense product ions m/z 125.098 and 123.082, respectively, in its MS/MS spectrum (Supplemental Fig. 4A). These characteristic losses together with high resolution mass of products and precursor ions suggested that this compound is an oxidized fatty acid with molecular formula $C_9H_{14}O_3$ (calc. m/z 169.087). The level of this compound increased 6.9 fold in the irradiated diet, compared to the control diet.

An analog of this compound with a difference of two mass units, i.e., m/z 171.103 (Rt 19.8 min) had product ions m/z 153.092, 127.113 and 125.098 due to losses of H_2O , CO_2 and $HCOOH$, respectively (Supplemental Fig. 4B). Inspection of its high resolution MS/MS spectra led to its assignment as a monohydroxynonenoic acid ($C_9H_{16}O_3$) (Table 1)

There were increased levels in the irradiated diet of a m/z 175.098 $[M-H]^-$ precursor ion which had the product ions m/z 130.965 and 129.092 due to neutral losses of CO_2 and $HCOOH$, respectively, in its MS/MS spectrum. These along with additional, abundant product ions m/z 75.011 and 57.001 suggested that this compound is a dihydroxy-octanoic acid ($C_8H_{16}O_4$) (calc. m/z 175.098).

Two nonanoic acid derivatives m/z 187.097 (Rt 16.0 min) and 189.113 (Rt 17.81 min) were identified with molecular formulae $C_9H_{16}O_4$ and $C_9H_{18}O_4$, respectively, based on interpretation of their MS/MS spectra. The ion m/z 187.097 $[M-H]^-$ yielded product ions m/z 169.087, 143.108 and 125.097 due to neutral losses of H_2O , CO_2 and H_2O plus CO_2 , respectively. The MS/MS fragmentation pattern of the ion m/z 187.097 matched well with the Metlin database MS/MS record for nonanedionic acid (calc. m/z 187.098).

The other ion m/z 189.113 $[M-H]^-$ showed losses of two molecules of H_2O (m/z 153.0930) and H_2O plus CO_2 losses giving rise to m/z 127.114. These data enabled us to propose the structure of this compound as either hydroxyl oxononenoic acid or dihydroxy-nonanoic acid (calc. m/z 189.113).

Two isobaric ions m/z 199.134 (Rt 18.93 min) and m/z 199.096 (Rt 14.19 min) both had losses of H_2O , $HCOOH$, and 72.021 Da, but with different ion intensities in their MS/MS spectra. The ion m/z 199.134 had weak product ions due to these losses, whereas m/z 199.096 showed significant product ions at m/z 181.086, 153.090, 127.113 and 125.063. In addition, it showed a prominent product ion m/z 171.102 $[M-HCO(CH_2)_7COO]^-$, indicating the presence a hydroxyl group at the C-9 position. Based on high resolution MS and MS/MS analyses, the ion m/z 199.134 was tentatively assigned as 9-hydroxy undecenoic acid

$C_{11}H_{19}O_3^-$ (calc. m/z 199.133). While the structure of the ion m/z 199.096 could not be proposed, it was an oxylipid with molecular formula $C_{10}H_{16}O_4$ (calc. m/z 199.097) (Table 1).

Another ion, m/z 209.118, whose intensity increased significantly on irradiation, had characteristic losses of oxidized fatty acids (H_2O , CO_2 and $HCOOH$), generating product ions m/z 191.106, 165.128 and 163.112, respectively. These data suggested it is a monohydroxy C_{12} unsaturated fatty acid with molecular formula $C_{12}H_{18}O_3$ (calc. m/z 209.118).

An ion m/z 231.160 that was three-fold lower in the irradiated diet had product ions in its MS/MS spectrum due to the losses of H_2O and $HCOOH$. Based on the accurate masses of its precursor and product ions, it corresponds to a molecular formula of $C_{12}H_{24}O_4$ (calc. m/z 231.160).

We also observed increased levels of oxidized long-chain fatty acids in nanoLC-MS analysis of the irradiated samples. An oxidized C_{18} fatty acid m/z 497.282 (Rt 20.93 min) was significantly increased in the irradiated diet and had an abundant product ion m/z 327.219, along with other product ions m/z 171.103, 155.108 and 137.097, in its MS/MS spectrum. Although precise structure of this compound is yet to be identified, $C_{18}:2-3O$ (m/z 327.2191) may be conjugated with $C_{8}:1-3O$, consistent with the molecular formula $C_{26}H_{42}O_9$ (calc. m/z 497.275).

An oxidized fatty acid m/z 217.108 (Rt 14.33 min) had product ions m/z 199.097, 171.103, and 155.108 due to neutral losses of H_2O , $HCOOH$, and CO_2 plus H_2O . Because there are two consecutive losses of CO_2 (one from m/z 199.097 and other from m/z 155.108) (Supplemental Fig. 5), it is proposed to be an oxidized dicarboxylic acid with molecular formula $C_{10}H_{18}O_5$ (calc. m/z 217.108) (Table 1).

Similarly, a deprotonated ion m/z 241.107 (Rt 17.67 min) also showed two consecutive losses of CO_2 yielding the product ion m/z 153.129. A combined loss of H_2O and CO_2 resulted in the product ion m/z 179.106. These data suggested that this compound is a C_{12} unsaturated fatty acid. The ionization of only one of the two acidic groups in C_3 - C_{12} dicarboxylic acids has been reported (13) and therefore, its formula was tentatively assigned as $C_{12}H_{18}O_5$ (calc. m/z 241.108) (Fig. 4A). The content of this compound was significantly higher (27-fold, $p = 0.0004$) in the irradiated diet, compared to the control diet (Table 1).

The ion m/z 259.118 showed the similar fragmentation pattern (the loss of two CO_2 and two H_2O molecules) suggesting this compound is an oxidized dicarboxylic fatty acid with molecular formula $C_{12}H_{20}O_6$ (calc. m/z 259.119) (Fig 4B).

Reactive aldehydes

Two closely eluting peaks (Rt 17.75 and 17.61 min) observed in positive ion data, m/z 155.106 $[M+H]^+$ and 173.117 $[M+H]^+$, respectively, were significantly up regulated in the irradiated diet, compared to the control diet (Table 1). The ion m/z 155.106 had a product ion m/z 137.095, due to the loss of H_2O . Further loss of CO (-27.994 Da) from this ion gave rise to m/z 109.102 (Fig. 5A). A prominent product ion m/z 67.056 ($C_5H_7^+$) was observed in

the MS/MS spectrum of the ion m/z 155.106. Interpretation of these data enabled putative identification of this compound as $C_9H_{14}O_2$, most likely 4-oxo-2-nonenal (calc. m/z 155.107) (Table 1).

The other ion m/z 173.117 showed similar fragmentation pattern as seen in the ion m/z 155.106 with mass difference due to loss of H_2O , possibly a hydroxylated oxononanal derivative with molecular formula $C_9H_{16}O_3$ (calc. m/z 173.118) (Fig. 5B).

Lipid analysis by MS/MS^{ALL}

MS/MS^{ALL} analysis was performed to evaluate changes in relatively hydrophobic lipid composition in diets after irradiation. Contents of linoleic and linolenic acids in foods changed significantly after irradiation. A marked decrease (~55%) in linoleic acid (the major precursor for the formation of reactive aldehyde compounds and oxylipins) was observed in the irradiated samples, based on their ion intensities (Supplemental Fig. 6). With the decrease in linoleic acid content, levels of oxylipins such as oxygenated products of linoleic acid increased in the irradiated samples.

An ion m/z 295.229 had a prominent product ion m/z 277.217 due to the loss of a water molecule in its MS/MS spectrum (Fig. 6A). Two diagnostic product ions of the epoxide of linoleic acid, m/z 155.144 and 171.102, were observed along with m/z 183.139 and 195.138 (14). Based on product ion information, the precursor ion m/z 295.229 was considered as a mono-oxygenated product of linoleic acid, probably a mixture of epoxides of linoleic acid – leukotoxin A (9,10 epoxy-12-octadecaenoic acid) and leukotoxin B (12,13 epoxy-9-octadecaenoic acid) or 9-and 13-hydroxy octadecadienoic acid (HODE) (elemental composition $C_{18}H_{32}O_3$, calc. m/z 295.227).

An increased level of another oxylipin m/z 293.211 was also observed in the lipid extract of the irradiated diet. The MS/MS spectrum of this ion contained the product ions m/z 275.203 and 249.223 with losses of H_2O and CO_2 , respectively (Fig. 6B). In addition to these ions, diagnostic product ions m/z 185.115 and 113.098 derived from 9-keto-octadecadienoic and 13-keto-octadecadienoic acids, respectively, were observed (15). These data indicated that the ion m/z 293.211 is a mixture of 9- and 13-keto-octadecadienoic acids (calc. m/z 293.212).

An oxygenation product of oleic acid (m/z 297.243) was significantly increased in the irradiated diet. The MS/MS spectrum of the ion m/z 297.243 contained the product ion m/z 279.231 along with other ions m/z 171.102, 155.106, 141.128, 183.011, and 127.112. These product ions are characteristic of oxygenation product of oleic acid (14). Based on these data, the ion m/z 297.243 could be a monooxygenation product with molecular formula $C_{18}H_{34}O_3$ (calc. m/z 297.244).

Irradiation also increased the levels of an ion m/z 311.223 which generated the product ions m/z 293.212, 267.192 and 275.201 due to neutral losses of H_2O , CO_2 and $2x H_2O$, respectively, characteristic of oxidized lipids. The presence of the product ions m/z 171.101, 139.113 and 113.099 in its MS/MS spectrum is consistent with molecular formula $C_{18}H_{32}O_4$ (calc. m/z 311.223), most likely an epoxyalcohol of octadecenoic acid.

Similarly, ions m/z 327.216 and 329.232 were observed in higher intensities in the irradiated samples, compared to the controls. The ion m/z 327.216 showed a similar MS/MS fragmentation pattern (m/z 309.209, 291.194, 281.247 and 267.233) to those of m/z 297.243 (m/z 279.231, 261.223, 251.238 and 237.217), with characteristic mass difference by 30, indicating the addition of CHOH in the structure of m/z 297.243 (Supplemental Fig. 7A). Thus, the ions m/z 327.216 was tentatively identified as oxidized C18-fatty acids with molecular formulae $C_{18}H_{32}O_5$ (calc. m/z 327.218). MS/MS of the ion m/z 329.232 produced characteristic fragment patterns including ions m/z 229.143, 211.133, 171.102 and 139.112 (Supplemental Fig. 7B), indicating this compound is trihydroxy-octadecenoic acid ($C_{18}H_{34}O_5$ calc. m/z 329.233). The structure is also supported by the published reference (16).

When precursor ions containing a product ion m/z 295.224 was search on the extracted sample to detect precursors of C18:2-O (15), a series of ions appeared in the mass range of 500–900 Da (Supplemental Fig. 8A). For example, m/z 577.481 is one of the most abundant ions and its MS/MS spectrum contained the prominent product ions m/z 541.367, 295.228 (deprotonated monohydroxy C18:2, calc. m/z 295.228), 281.248 (deprotonated C18:1, calc. m/z 281.248) and 279.233 (deprotonated C18:2, calc. m/z 279.232) due to the neutral losses of 36.114, 282.225, 296.233 and 298.248 Da, respectively (Supplemental Fig. 8B). These data suggested that the ion m/z 577.482 is a mixture of oleic acid-dihydroxy oleic acid and linoleic acid-dihydroxy stearic acid with the molecular formula $C_{36}H_{66}O_5$ (calc. m/z 577.484). Structurally, they are likely to be similar to previously reported branched fatty acid esters of hydroxy fatty acids (FAHFAs) (17).

Further oxidation of this compound may give rise to another novel FAHFA with m/z 593.473 which had product ions m/z 281.247 (C18:1) and 311.223 (C18:2–2O) in its MS/MS spectrum, consistent with molecular formula $C_{36}H_{66}O_6$ (calc. m/z 593.479). We also detected m/z 625.469 showing a product ion m/z 589.451 due to the loss of $2xH_2O$ in its MS/MS spectrum. The presence of product ions m/z 327.217 (calc. m/z 327.218) and the ion m/z 297.244 indicated this compound to be an oxidized lipid with molecular formula $C_{36}H_{66}O_8$ (calc. m/z 625.469).

An ion m/z 487.328 $[M-H]^-$ showed prominent product ions m/z 451.350, 329.232, 281.247, 279.233, 171.103 and 159.102 in its MS/MS spectrum (Supplemental Fig. 9). The presence of product ions m/z 329.232 and m/z 171.103 were indicative of an oxidized C_{18} fatty acid. The observed accurate mass of the deprotonated molecular ion and MS/MS product ions, the molecular formula of this compound is proposed to be $C_{26}H_{48}O_8$ (calc. m/z 487.328). This is a novel lipid consisting of an oxidized long-chain fatty acid (C18:O-3O) esterified with medium chain fatty acid (C8:1–2O).

Discussion

Several changes in the profiles of dietary metabolites and lipids were observed in the irradiated samples. There was a significant difference in the content of phytochemicals between the irradiated and the control diet samples. Metabolomics data and pathway analyses are consistent with radiation-induced increases in oxidized forms of linoleate. A

limitation of all these procedures was the formation of novel oxidized lipid metabolites not recognized by the databases.

We observed the marked increase in extractable glucosinolates in the irradiated diets. Glucosinolates are sulfur and nitrogen containing glycosides, widely found in cruciferous vegetables (10). Their hydrolyzed products, isothiocyanates, are well known chemopreventive agents against the development of cancers (18). Effect of radiation on glucosinolates of cabbage has previously been reported (19). The increased levels of glucosinolates in the irradiated diets may be responsible for breast cancer chemoprevention in rats, as the number of mammary tumors in rats were 40% less than when using non-irradiated diets (Clinton Grubbs, unpublished results).

Up-regulation of glucosinolates upon irradiation may be possible through inactivation of myrosinase enzyme, causing intact glucosinolates to remain in the diet. Other phytochemicals such as polyphenol glycosides containing a malonyl or hydroxyl methyl-glutaryl moieties were significantly increased in the irradiated diet. Gamma rays can also penetrate cells and may cause the breakdown of middle lamella in cell wall thereby releasing these compounds (20,21). This suggests that gamma irradiation may alter the pharmaceutical state of solid food.

We observed significant differences in the levels of PUFAs and their oxygenation products between the control and irradiated diet. Linoleic acid and α -linolenic acid are the main polyunsaturated fatty acids in plant-based diets. Irradiation of diet will lead to formation of reactive oxygen species that promote lipid oxidation in foods rich in these fatty acids (22). Oxylipins are a diverse family of oxygenated fatty acids that are produced by oxidative metabolism, either enzymatic or non-enzymatic of polyunsaturated fatty acids such as linoleic acid and linolenic acid (22). Chemically-derived, racemic mixtures of oxylipins are formed and may be esterified with other oxidized lipids.

In our study, irradiation increased the content of a number of oxidized lipids, particularly, linoleic acid-derived oxidation products containing multiple combination of hydroxyl, epoxide, and or ketone moieties. A mixture of 13- and 9- HODE is one of the possibilities which is synthesized *in vivo* from 18:2 released from the endogenous triacylglycerol pool (23). Effects of storage and Gamma-irradiation on metabolic pattern, including linoleic acid metabolism in red blood cells has previously been reported (24).

We also observed a series of novel oxidized lipids with a branched ester linkage between a fatty acid and a hydroxyl fatty acid or oxidized lipids (Supplemental Fig. 8 and 9). While biology of these lipids are unknown, branched fatty acid esters of hydroxyl fatty acids, such as palmitic-acid-9-hydroxy-stearic acid, are implicated in insulin sensitivity and are found in reduced levels in adipose tissue and serum of insulin-resistant humans (17). Although previously reported as endogenous lipids, our results demonstrate that irradiation increases levels of branched fatty acid esters of hydroxylated fatty acids in the diet as well. Emerging studies have shown that oxylipins derived from linoleic acid can affect a broad range of physiological effects such as inflammation, cell proliferation and vascular function (25) and these may be implicated in cancer risk.

While infection, wounding and stress are known to induce oxidized lipids by biochemical enzymatic processes (26), effects of radiation on oxylipin formation in diet is unknown. Dietary lipid oxidation is one of the major problems in maintaining food quality (27). While *in vitro* studies on biological effects of individual oxylipins have been reported, effects of dietary oxylipins and or other oxidized lipids which exist together with many other nutrients and phytochemicals on health and diseases such as breast cancer are yet to be evaluated *in vivo*.

Conclusions

Our studies clearly indicate that Cobal-60 irradiation induces modifications of animal diet components in terms of content and or chemical structures. Several peaks corresponding to phytochemicals and oxidized fatty acids of animal diet that had been modified (mostly upregulated) by irradiation and they were identified at the level of their molecular formulae. However, the biological significance of the food metabolite modification after irradiation is still unknown. Treatment of rodent diets with Gamma-irradiation is not benign. Despite reducing the microbiome content of the food, it leads to increased oxidation of lipids and altered component availability. Surprisingly, it led to a reduction in carcinogenesis in a rodent model of breast cancer. Future studies will examine which of the radiation generated-components contribute to the changes in cancer risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Volcano plot in negative ion mode showing the statistical significance (y axis) and fold change (x axis) for difference between irradiated and non-irradiated diets.

Fig. 2. Score plots of principle component analysis from control (NR) and irradiated (IR) samples in positive [A] and negative ion [B] modes. These plots display a clear separation between irradiated and control diet samples.

Fig. 3.
nano-LC-MS/MS spectrum of m/z 591.136 $[M-H]^-$ (Rt 14.92 min).

Fig. 4. ESI-MS/MS spectra of oxidized lipids [A] m/z 241.107; [B] m/z 259.118 in negative ion mode.

Fig. 5.
ESI-MS/MS spectra oxidized lipids [A] m/z 155.106; [B] m/z 173.117 in positive ion mode.

Fig. 6. ESI-MS/MS spectra of oxidized lipids [A] m/z 295.229; [B] m/z 293.213 in negative ion mode.

Table 1

Metabolite ions undergoing significant changes in irradiated diet

Ionization (-)	Standard deviation	Retention time (min)	p-value	Experimental (<i>m/z</i>)	Empirical formula	Calculated (<i>m/z</i>)
Fold change						
3.00 up	0.136	14.20	0.0001	129.054	C ₆ H ₁₀ O ₃	129.0557
2.73 up	0.078	7.24	0.00003	147.029	C ₅ H ₈ O ₅	147.0299
1.31 up	0.019	11.95	0.00009	159.067	C ₇ H ₁₂ O ₄	159.0657
1.59 up	0.073	14.27	0.0124	159.103	C ₈ H ₁₆ O ₃	159.1021
6.9 up	0.980	20.87	0.0267	169.087	C ₉ H ₁₄ O ₃	169.0870
1.5 down	0.051	19.87	0.0025	171.103	C ₉ H ₁₆ O ₃	171.1027
1.8 up	0.023	14.93	0.00005	175.098	C ₈ H ₁₆ O ₄	175.0976
2.11 up	0.065	16.02	0.00007	187.097	C ₉ H ₁₆ O ₄	187.0976
3.74 up	0.223	17.81	0.00025	189.113	C ₉ H ₁₈ O ₄	189.1132
2.49 up	0.036	18.93	0.000002	199.134	C ₁₁ H ₂₀ O ₃	199.1340
4.22 up	0.150	14.19	0.00003	199.096	C ₁₀ H ₁₆ O ₄	199.0970
3.46 up	0.159	20.70	0.00010	209.118	C ₁₂ H ₁₈ O ₃	209.1183
4.56 up	0.128	14.33	0.00001	217.108	C ₁₀ H ₁₈ O ₅	217.1081
3.0 up	0.039	15.70	0.00057	231.160	C ₁₂ H ₂₄ O ₄	231.1602
27.4 up	0.652	17.67	0.000002	241.107	C ₁₂ H ₁₈ O ₅	241.1081
2.83 up	0.091	14.85	0.000036	259.118	C ₁₂ H ₂₀ O ₆	259.1187
2.03 up	0.064	10.75	0.00009	299.080	C ₁₃ H ₁₆ O ₈	299.0772
2.49 up	0.048	12.62	0.000005	357.082	C ₁₅ H ₁₈ O ₁₀	357.0827
2.79 up	0.055	10.84	0.000006	417.106	C ₁₇ H ₂₂ O ₁₂	417.1038
13.0 up	0.393	8.07	0.000007	424.038	C ₁₄ H ₁₉ NO ₁₀ S ₂	424.0378
3.27 up	0.264	20.93	0.00099	497.281	C ₂₃ H ₄₂ O ₉	497.2751
2.80 up	0.196	12.33	0.00079	508.103	C ₁₆ H ₃₁ NO ₁₁ S ₃	508.0986
6.00 up	0.092	13.82	0.000007	522.111	C ₁₇ H ₃₂ O ₁₁ NS ₃	552.1143
2.36 up	0.193	15.48	0.0022	536.130	C ₁₈ H ₃₅ NO ₁₁ S ₃	536.1299

Ionization (–)	Standard deviation	Retention time (min)	p-value	Experimental (<i>m/z</i>)	Empirical formula	Calculated (<i>m/z</i>)
Fold change						
2.89 up	0.060	14.92	0.0000006	591.136	C ₂₇ H ₂₈ O ₁₅	591.1350
Ionization (+)						
5.74 up	0.187	17.75	0.0026	155.106	C ₉ H ₁₄ O ₂	155.1067
5.90 up	0.748	17.61	0.0028	173.117	C ₉ H ₁₈ O ₃	173.1172
3.62 up	0.505	14.29	0.0065	545.130	C ₂₆ H ₂₄ O ₁₃	545.1290

The standard deviation was calculated from the pooled variance of the irradiated and non-irradiated groups which was then divided by the mean of the non-irradiated group. The p-value was generated by using Student's T-test.