

Ketogenic Diets Enhance Oxidative Stress and Radio-Chemo-Therapy Responses in Lung Cancer Xenografts

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Abstract

Purpose: Ketogenic diets are high in fat and low in carbohydrates as well as protein which forces cells to rely on lipid oxidation and mitochondrial respiration rather than glycolysis for energy metabolism. Cancer cells (relative to normal cells) are believed to exist in a state of chronic oxidative stress mediated by mitochondrial metabolism. The current study tests the hypothesis that ketogenic diets enhance radio-chemo-therapy responses in lung cancer xenografts by enhancing oxidative stress.

Experimental Design: Mice bearing NCI-H292 and A549 lung cancer xenografts were fed a ketogenic diet (KetoCal 4:1 fats: proteins+carbohydrates) and treated with either conventionally fractionated (1.8–2 Gy) or hypofractionated (6 Gy) radiation as well as conventionally fractionated radiation combined with carboplatin. Mice weights and tumor size were monitored. Tumors were assessed for immunoreactive 4-hydroxy-2-nonenal-(4HNE)-modified proteins as a marker of oxidative stress as well as proliferating cell nuclear antigen (PCNA) and γH2AX as indices of proliferation and DNA damage, respectively.

Results: The ketogenic diets combined with radiation resulted in slower tumor growth in both NCI-H292 and A549 xenografts ($P < 0.05$), relative to radiation alone. The ketogenic diet also slowed tumor growth when combined with carboplatin and radiation, relative to control. Tumors from animals fed a ketogenic diet in combination with radiation showed increases in oxidative damage mediated by lipid peroxidation as determined by 4HNE-modified proteins as well as decreased proliferation as assessed by decreased immunoreactive PCNA.

Conclusions: These results show that a ketogenic diet enhances radio-chemo-therapy responses in lung cancer xenografts by a mechanism that may involve increased oxidative stress. *Clin Cancer Res*; 19(14); 3905–13. ©2013 AACR.

Introduction

Over the past two decades, there has been little improvement in the overall survival trends in patients with locally advanced lung cancer despite the development of new chemotherapy agents and advances in immunotherapy. Therefore, complementary approaches that enhance the efficacy of radiation and chemotherapy while causing minimal toxicity would have a high therapeutic benefit.

It has long been known that malignant cells have high levels of glucose uptake, glycolysis, and pentose phosphate activity even in the presence of oxygen. This glycolytic

phenotype has been associated with higher grade lung neoplasms and poorer prognosis (1), while reversing this glycolytic phenotype using biochemical or pharmacologic approaches has resulted in decreased metastases and tumor growth (2). Cancer cells also have alterations in their mitochondrial structure and function resulting in increased levels of reactive oxygen species (ROS) such as O_2^- and H_2O_2 , relative to normal cells (3–5). It has been proposed with significant supporting data that cancer cells use increased glucose metabolism to generate reducing equivalents that are necessary to facilitate decomposition of ROS as an adaptive response to metabolic oxidative stress caused by cancer cell-specific dysfunctional mitochondrial O_2 metabolism (3, 6–8). Therefore, approaches that further increase mitochondrial oxidative metabolism, while limiting glucose metabolism, may selectively increase oxidative stress in cancer cells resulting improved responses to radiation and chemotherapy. One clinical approach that exploits mitochondrial metabolism is a ketogenic diet.

Ketogenic diets typically consist of 90% fat, 8% protein, and 2% carbohydrate and are an established therapy for epilepsy (9). Ketogenic diets derive their name from the generation of ketones, mainly beta-hydroxybutyrate (β HB) and acetoacetate (AA) which occur due to increased fatty

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

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doi: 10.1158/1078-0432.CCR-12-0287

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Translational Relevance

Ketogenic diets are high in fat, low in carbohydrates, and are well established as an alternative therapy for childhood epilepsy. This report shows that a ketogenic diet enhances radio-chemo-therapy responses as well as enhancing oxidative stress in human lung cancer xenografts. As ketogenic diets are an established therapy in humans, these studies may be rapidly translated into the clinical setting, potentially allowing for improved cancer control without added normal tissue toxicity.

acid oxidation in the liver and are precursors for acetyl CoA, the first step in the citric acid cycle (9). Because of the low carbohydrate content of ketogenic diets, there is often a reduction of blood glucose levels and overall greater glycemic control (10, 11). It has also been shown that the rates of glucose uptake as well as rates of flux into the glycolytic pathway are reduced in rats fed a ketogenic diet (12, 13). The combination of reduction in blood glucose and rise in ketones is believed to force cells to rely more heavily on mitochondrial respiration than on glycolysis for energy metabolism (14).

The current study tests the hypothesis that feeding a ketogenic diet to mice bearing lung cancer xenografts induces oxidative stress in tumors and improves responses to radio-chemo-therapy. The results show that ketogenic diets effectively enhance tumor growth inhibition and prolong animal survival in response to radio-chemo-therapy in mice with lung cancer xenografts by a mechanism that seems to involve oxidative stress. Furthermore, ketogenic diets effectively enhanced responses in lung cancer xenografts using both conventional (1.8–2 Gy) and hypofractionated (6 Gy) radiation dosing schemes without causing increased systemic toxicity as indicated by changes in animal weight or behavior. These data support the hypothesis that ketogenic diets may enhance lung cancer responses to radio-chemo-therapy by a mechanism that involves selective enhancement of oxidative stress in tumor tissues.

Materials and Methods

Cell and culture conditions

A549 and H292 cells were obtained from the American Type Culture Collection. A549 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10%FBS (Hyclone) and 0.01% gentamycin sulfate (Cellgro). H292 cells were maintained in RPMI containing 10% FBS (Hyclone) and 0.01% gentamycin sulfate (Cellgro). Glucose-depleted media consisted of DMEM devoid of glucose and pyruvate but containing glutamine (Gibco) to which 70 mg/dL D-glucose was added.

Clonogenic survival analysis

A549 or H292 cells (120,000/60 mm dish) were plated and allowed to grow in their respective tissue culture media for 24 hours. All plates were then placed into DMEM

glucose-depleted media with 70 mg/dL glucose added back. Selected tissue culture dishes were supplemented with 3 mmol/L (R)-(β)-3-hydroxybutyric acid sodium salt (βHB; Sigma Aldrich) and 1.5 mmol/L lithium AA (Sigma Aldrich) mimicing the maximum concentration and approximate ratio of ketones found in the blood of adult humans on a ketogenic diet (14). After 24 hours of ketone treatment, cultures were exposed to sham, 0.5 Gy, 1 Gy, 2 Gy, 3 Gy, or 4 Gy ionizing radiation after which clonogenic survival analysis was conducted as previously described in standard complete media (15).

Tumor xenograft growth

Female 4 to 6-week-old athymic-nu/nu mice were purchased from Harlan Laboratories. Mice were housed in the Animal Care Facility at the University of Iowa (Iowa City, IA) and all procedures were approved by the University of Iowa Institutional Animal Care and Use Committee and conformed to NIH guidelines. H292 and A549 tumor cells were injected subcutaneously into the right flanks as previously described (15). Treatment began when tumor volumes measured approximately 30 mm³.

Ketogenic diet and ketone monitoring

The mice in the ketogenic diet groups were fed *ad libitum* KetoCal (Nutricia North America, a formula designed for children with epilepsy). This diet has a ketogenic ratio (fats: proteins + carbohydrates) of 4:1 (energy distribution: fat 90%, carbohydrate 1.60%, and protein 8.40%) and the fat is derived from soybean oil (100% long chain fatty acids; 22% saturated fat, 24% monounsaturated fat, and 15% polyunsaturated fat). KetoCal was prepared as a paste on a culture-dish lid by adding water (water: KetoCal 1:2) then placed upside down in the food hopper and attached to assure the animals access (16). Control mice were fed a standard rodent diet (NIH-31 modified 25% protein, 21% fat, and 54% carbohydrate). Ketogenic diet was started 2 days before the initiation of ionizing radiation and continued for 24 hours after the last treatment. Blood βHB and glucose levels were measured using the Precision Xtra (Abbott Laboratories) machine via a tail vein stick. Previous results indicated that the average blood levels of βHB starting 3 days after beginning the ketogenic diet and continuing through the end of treatment were 1.01 ± 0.64 mmol/L for the ketogenic diet mice versus 0.18 ± 0.08 mmol/L for control mice (17).

Ionizing radiation *in vivo*

Mice in all treatment and sham groups were anesthetized using 87.5 mg/kg ketamine and 12.5 mg/kg xylazine mixture in the radiation facility at the University of Iowa. The mice in the ionizing radiation groups were placed in a lead box with only the right flank exposed. Radiation was delivered using a Pantak Therapax DXT 300 X-ray machine operated at 200 kVp with added filtration of 0.35 mm Cu + 1.5 mm Al, resulting in a beam quality of 0.95 mm Cu. Tumors were measured daily using Vernier calipers (volume = (length × width²) / 2) and euthanized when tumor length exceeded 1.5 cm in any dimension. For the 12 Gy total

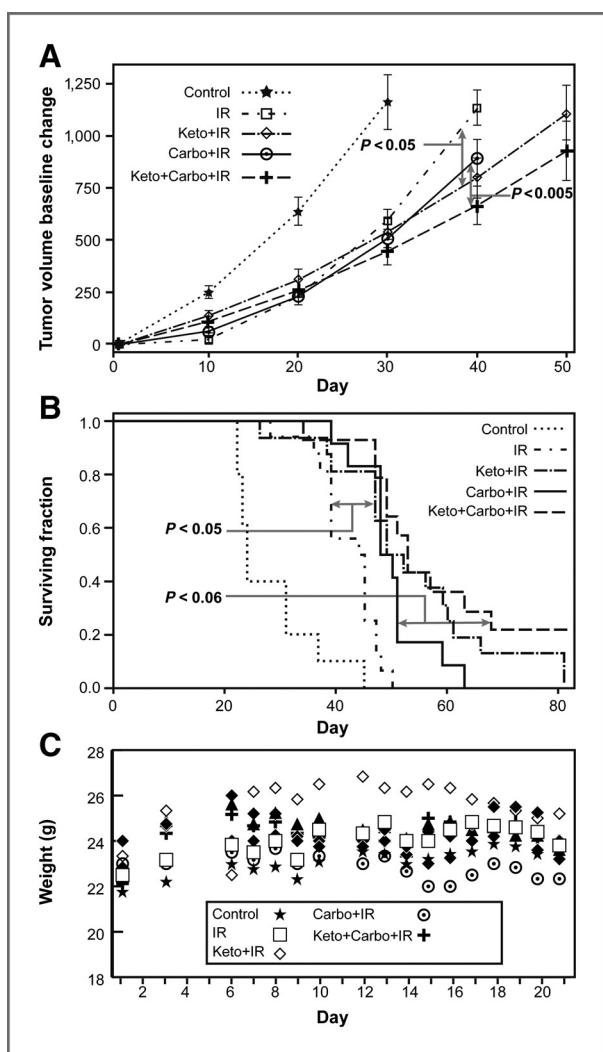


Figure 1. The ketogenic diet (Keto) enhances chemo-radiation responses in H292 lung cancer xenografts. Nude mice (5–16 animals per group) were injected with H292 cells in the flank and tumors allowed to grow to 30 mm^3 . Treated mice were given $3 \times 15 \text{ mg/kg}$ carboplatin doses on 3 consecutive Mondays. Following each of the first 2 doses of carboplatin the mice were irradiated with $3 \times 2 \text{ Gy}$ ionizing radiation (IR) fractions Monday, Wednesday, and Friday for 2 weeks (12 Gy total dose) followed by 1 final carboplatin dose on the following Monday. The ketogenic diet started 2 days before the first chemo-radiation dose and continued until 2 days following the last dose for a total of 16 days. When maximum tumor diameter exceeded 1.5 cm , mice were sacrificed. A, tumor volume growth curve estimates using mixed linear regression analysis show that ketogenic diet significantly ($P < 0.05$) decreases tumor growth rates when combined with ionizing radiation and carboplatin-ionizing radiation, relative to ionizing radiation and carboplatin-ionizing radiation alone, respectively. B, pairwise group comparisons of Kaplan-Meier survival curves show that ketogenic diet significantly enhances ionizing radiation response ($P < 0.05$), relative to ionizing radiation alone and approaches significance for carboplatin-ionizing radiation sensitivity ($P < 0.06$). C, pairwise group comparisons of animal weights show that all treatments were well tolerated as shown by a lack of significant weight change (errors omitted for clarity).

dose-fractionated ionizing radiation experiment (Fig. 1), mice were divided into the following treatment groups (N represents the total number of animals per treatment

group combined from 2 duplicative experiments); (i) control group ($N = 10$), (ii) ketogenic diet group ($N = 12$), (iii) ionizing radiation group (12 Gy total dose in $6 \times 2 \text{ Gy}$ fractions every other day for 2 weeks; $N = 16$), (iv) ionizing radiation + ketogenic diet group ($N = 16$), (v) carboplatin (15 mg/kg intraperitoneal once per week $\times 3$ doses; $N = 6$), (vi) carboplatin + ketogenic diet ($N = 5$), (vii) ionizing radiation + carboplatin ($N = 12$), and (viii) ionizing radiation + carboplatin + ketogenic diet ($N = 14$). For the 61.2 Gy total dose-fractionated ionizing radiation experiment (Fig. 2) mice were divided into 4 groups; (i) Control ($N = 6$), (ii) ketogenic diet ($N = 7$), (iii) ionizing radiation (61.2 Gy in 34 doses, 1.8 Gy every other day; $N = 8$], and (iv) ketogenic diet + ionizing radiation ($N = 7$). The 4 treatment groups for the 12 Gy hypofractionation ionizing radiation experiment (Fig. 3) with 9 mice per treatment group were; (i) Control, (ii) ketogenic diet, (iii) ionizing radiation (12 Gy in $2 \times 6 \text{ Gy}$ fractions, 48 hours apart), and (iv) ketogenic diet + ionizing radiation. At the completion of

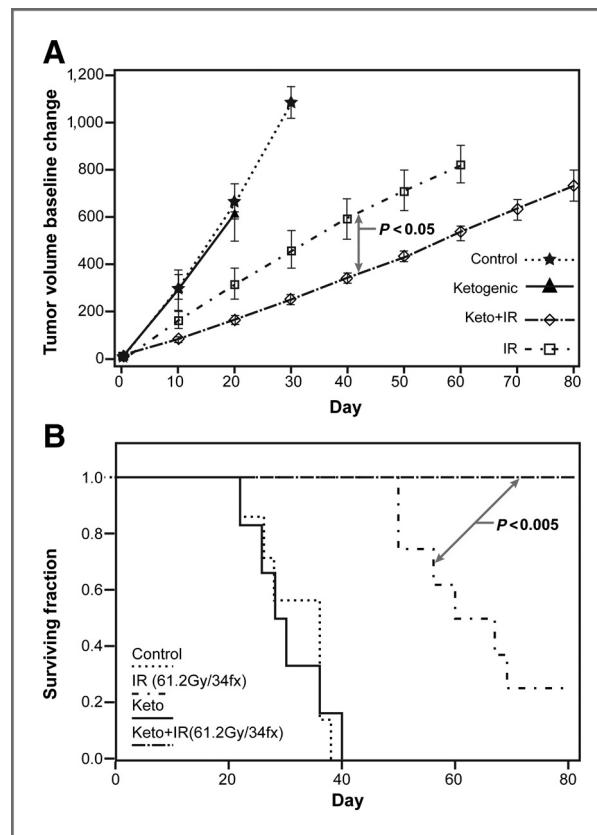


Figure 2. A protracted ketogenic diet increases radiation response in H292 lung cancer xenografts given conventional fractionated radiation. H292 xenografts were grown in nude mice as in Fig. 2. Mice (6–8 per group) were treated with ketogenic diet for the entire treatment time and/or a 61.2 Gy total dose of radiation in $34 \times 1.8 \text{ Gy}$ fractions 3 times per week (M, W, F). The experiment was terminated 81 days after the beginning of ketogenic diet. A, tumor growth curve estimates showed that the ketogenic diet significantly enhances radiation response as assayed by tumor growth rates ($P = 0.0210$). B, pairwise group comparisons of Kaplan-Meier survival curves also showed that the ketogenic diet significantly enhanced radiation response ($P = 0.0041$).

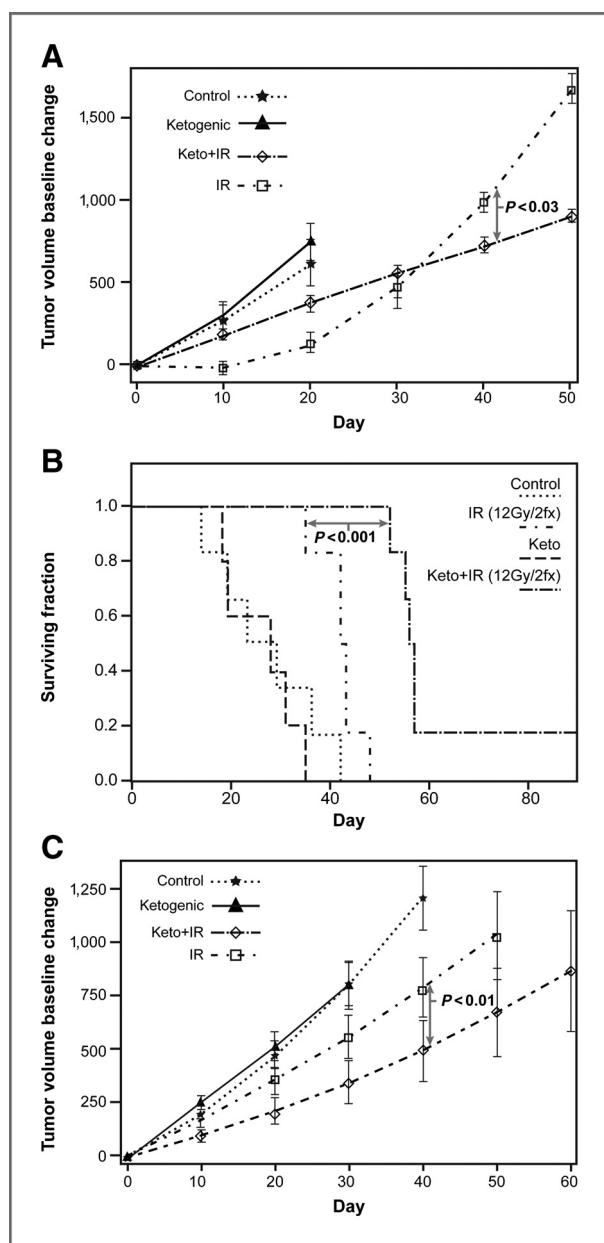


Figure 3. The responses of H292 and A549 lung cancer xenografts to hypofractionated radiation were enhanced by the ketogenic diet. H292 and A549 xenografts were grown in nude mice as in Fig. 2. Mice (5–9 per group) were treated with ketogenic diet for 2 days before radiation and continuing for a total of 7 days. Hypofractionated radiation (2×6 Gy fractions) was delivered on day 3 and 5 of the protocol. Tumor growth curves (A) and Kaplan-Meier survival plots (B) showed enhanced radiation responses in animals with H292 xenografts fed the ketogenic diet ($P < 0.05$), relative to ionizing radiation alone. C, tumor volume growth curve estimates of A549 xenografts also showed that ketogenic diet + ionizing radiation significantly slowed tumor growth when compared with radiation alone ($P < 0.05$). All treatments were well tolerated as shown by the lack of weight loss during therapy (Supplementary Figs. S1A and S1B).

the ketogenic diet, the 3 mice with the largest tumors from each treatment group were collected and frozen in liquid nitrogen for oxidative stress assessment.

4-Hydroxy-2-nonenal-(4HNE)-modified protein immunoblotting assay

Approximately 20 mg of mouse tumor was washed and homogenized in 300 μ L 500 mmol/L potassium phosphate, 50 mmol/L EDTA buffer pH 7.0 with Complete Mini-protease inhibitor (Roche Diagnostics). Protein concentrations were determined by Lowry Assay. Twenty-five micrograms of protein was placed in an equal volume with nanopure H_2O and blotted onto prewetted Sequi-Blot polyvinylidene difluoride (PVDF) membrane (Bio-Rad) and allowed to dry. After rewetting in methanol, the membrane was incubated in 250 mmol/L sodium borohydride in 100 mmol/L MOPS, pH 8.0 for 15 minutes to chemically reduce the Schiff base adduct to reveal the Michael addition product for antibody recognition. The membrane was then washed 3 times each with nanopure H_2O followed by PBS, then blocked for 30 minutes in 2% albumin in PBS + 1% Tween 20. The blot was incubated with the primary antibody recognizing the Michael addition product of 4HNE-modified cellular proteins (18) diluted 1/2,000 overnight at 4°C, followed by 2 hours in secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit polyclonal antibody (1/20,000), and chemiluminescence detection (ECL Plus Western Blotting Detection System, GE Healthcare) with X-ray film. Immunoreactive protein on the dot blot was analyzed using integrated densities determined using ImageJ software.

Western blot assays

Approximately 100 mg piece of tumor was homogenized in 0.5 mL of ice-cold Tris buffer pH 7.8 with Complete Mini-protease inhibitor and PhosStop phosphatase inhibitor cocktail (Roche Diagnostics). After a freeze/thaw, the samples were centrifuged at 10,000 $\times g$ for 15 minutes at 4°C. Protein concentrations were determined by Lowry method. Proliferating cell nuclear antigen (PCNA) Western blot analysis was carried out using a monoclonal anti-PCNA clone PC10 antibody (M0879, Dako) running both 10 and 70 μ g of protein per lane on separate occasions, electrophoresed on 4% to 20% gradient SDS-polyacrylamide gels. Bands were detected using ECL Plus Western Blotting Detection System (GE Healthcare) with detection by either X-ray film and/or a Typhoon FLA 7,000 fluorescent detection system. Band-integrated densities were determined with ImageJ software and averaged between the two gels. The γ H2AX Western was conducted following the same procedure described for PCNA but running on a 15% SDS-polyacrylamide gel using reaction with the polyclonal anti-phos-H2AX Ser 139 antibody (#07-164, Upstate Biotechnology).

Statistical analysis

The analysis of *in vivo* results focused on treatment group comparisons of tumor growth and survival. Regression analysis was used to model tumor growth as a non-linear function of follow-up time and to make treatment group comparisons. Within-animal correlation structures were included in the models to account for repeated

measurement of tumor size over time. Plots of the estimated tumor means and their standard errors are provided in the results section. Estimates of survival were obtained with the methods of Kaplan-Meier and compared with log-rank tests. All associated statistical tests were 2-sided and assessed for significance at the 5% level with the SAS statistical software.

Results

Ketogenic diet enhances radio-chemo-therapy responses in mouse non-small-cell lung carcinoma xenografts

Athymic nude mice were injected with $2-3 \times 10^6$ H292 non-small-cell lung carcinoma (NSCLC) cells in their right flank. When tumors reached approximately 4 mm in diameter, mice were treated with ionizing radiation alone or ionizing radiation combined with: ketogenic diets, carboplatin, and/or standard fractionated ionizing radiation doses (12 Gy in 6×2 Gy fractions). All animals consuming a ketogenic diet for 48 hours or more had serum ketone levels more than 0.3 mEq/L (or 0.3 mmol/L) and were considered to be in ketosis as per the Emory Warner Clinical Laboratories of the University of Iowa Hospitals and Clinics. Consistent with previous studies (17), the mice eating the ketogenic diets showed blood β HB levels more than 0.3 mEq/L (mean = 1.4 ± 0.8 mEq/L) throughout the treatment period, whereas mice treated with standard mouse chow were consistently less than 0.3 mEq/L (mean 0.1 ± 0.1 mEq/L).

Mice treated with ketogenic diet + ionizing radiation or ketogenic diet + ionizing radiation + carboplatin (12 Gy in 6×2 Gy fractions ionizing radiation) showed a significant decrease in tumor growth rate compared with mice treated with identical therapies on standard chow ($P = 0.0470$ and $P = 0.0046$, respectively; Fig. 1A). Furthermore, mice treated with ketogenic diet + ionizing radiation also showed a significant survival advantage over mice treated with ionizing radiation alone ($P = 0.0281$); survival of mice treated with ketogenic diet + ionizing radiation + carboplatin versus ionizing radiation + carboplatin was also prolonged ($P = 0.0595$) but did not reach less than 0.05 significance level (Fig. 1B). All treatments were well tolerated as shown by a lack of weight loss as well as general condition and activity of the mice (Fig. 1C). These data support the conclusion that ketogenic diets can effectively enhance responses to radio-chemo-therapy in animals bearing NSCLC xenografts without causing overt signs of toxicity.

Ketogenic diets enhance responses of lung cancer xenografts to a clinically relevant radiation dose fractionation scheme

To replicate the duration and dosing of common human lung cancer-fractionated radiotherapy protocols, mice bearing H292 xenografts fed a ketogenic diet or control diet were treated with a total dose of 61.2 Gy in 34×1.8 Gy fractions (Fig. 2). Mice consuming a ketogenic diet had achieved an average blood β HB level of 1.4 ± 0.4 mmol/L by the first day of ionizing radiation treatment. Because of the more protracted radiation exposure regimen, some mice lost a significant amount of weight (15% of starting weight) by the end of the third or fourth week of treatment. This weight loss was also associated with hyperkeratotic dermatitis likely caused by coryneform bacterium, which is a common pathogen in athymic nude mice. The weight loss was managed by feeding the control mice with a diet supplement (2019 Teklad) consisting of approximately the same fat, carbohydrate, and protein as the standard diet, but soaked in water. Mice on ketogenic diets were supplemented with KetoCal 3:1 formula every other day for 2 weeks and the KetoCal 4:1 liquid formula. Ketone levels were measured before each ionizing radiation dose to verify that the mice were maintained in ketosis. Mice were sacrificed once tumors reached 1.5 cm in diameter or the day following the completion of 34 ionizing radiation fractions. None of the mice in the ketogenic diet + ionizing radiation group reached criteria to be sacrificed at the completion of ionizing radiation treatment. Mice treated with the ketogenic diet in combination with ionizing radiation showed significantly slower tumor growth ($P = 0.0210$; Fig. 2A) and prolonged survival ($P = 0.0041$), relative to ionizing radiation alone (Fig. 2B). These results clearly show that a prolonged ketogenic diet combined with a clinically relevant ionizing radiation dose fractionation schedule is well tolerated and enhances radiation response in NSCLC xenografts.

Ketogenic diets enhance responses of lung cancer xenografts to hypofractionated radiation

Hypofractionated radiation therapy (delivering higher doses in fewer fractions) has become of increasing interest with the recognition of a potential improvement in the therapeutic ratio between the tumor volume and normal anatomic structures. Clinically, hypofractionation allows for increased patient convenience and reduced costs without sacrificing efficacy and is used to treat early-stage lung cancers (19). In addition, hypofractionated radiation shortens the duration of radiation therapy which would potentially increase patient compliance with the diet.

For hypofractionated radiation experiments, H292 and A549 xenografts were grown in nude mice as in Figs. 1 and 2. Mice were fed a ketogenic diet for a total of 7 days starting 2 days before and continuing 2 days after 2 fractions of 6 Gy ionizing radiation (12 Gy total dose). Both H292 and A549 xenograft bearing animals treated with ketogenic diet in combination with hypofractionated ionizing radiation showed significantly slower tumor growth rates, relative to ionizing radiation alone ($P = 0.0299$ and $P = 0.0054$, respectively; Fig. 3A and C). H292 xenografts treated with ketogenic diet + ionizing radiation also showed a survival advantage compared with those treated with ionizing radiation alone ($P = 0.0006$; Fig. 3B) but this was not seen in A549 xenografts (data not shown). All treatments were well tolerated as shown by a lack of weight loss and general

condition of the mice (Supplementary Fig. S1A and S1B). These results show that a ketogenic diet enhances responses of NSCLC xenografts to hypofractionated radiotherapy.

Ketogenic diets in combination with ionizing radiation increase immunoreactive 4HNE-modified proteins in tumor tissue

To assess oxidative stress *in vivo*, immunoreactive 4-hydroxy-2-nonenal-(4HNE)-modified protein was analyzed in tumors as a marker of oxidative protein damage caused by lipid peroxidation. Dot blot analysis of tumor samples harvested at the end of the hypofractionated-ionizing radiation experiment shown in Fig. 3A and B showed increased 4HNE-modified protein in H292 xenografts treated with ketogenic diets + hypofractionated ionizing radiation versus hypofractionated ionizing radiation alone ($P < 0.05$; Fig. 4A and B). These data support the hypothesis that a ketogenic diet combined with hypofractionated ionizing radiation causes an increase in oxidative damage to proteins

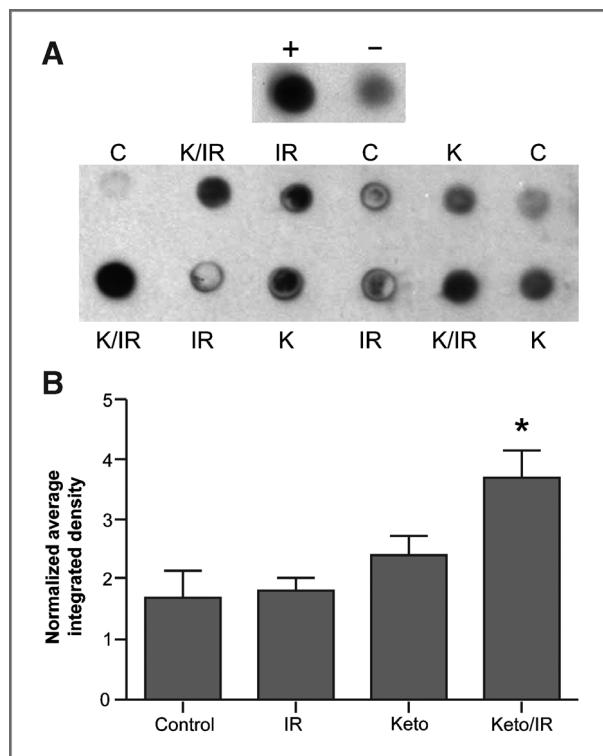


Figure 4. Ketogenic diets combined with radiation increases 4HNE-modified proteins in H292 mouse lung cancer xenografts. Equal protein from H292 xenograft tumor homogenates taken from animals treated as in Fig. 3 ($N = 3$ from each group) were blotted onto PVDF membranes and stained with polyclonal antibody against 4HNE-modified proteins. This analysis was repeated twice and a representative blot is shown in A. Positive (+) and negative (-) controls represent the immunoreactivity derived from H292 cell homogenates treated with and without 100 μ mol/L genuine 4HNE for 1 hour at 37°C. B, quantification of dot blots by Image J analysis and samples were normalized to the background on each blot. Error bars represent ± 1 SEM. One way ANOVA with Newman-Keuls Multiple Comparison Test showed the ketogenic diet + ionizing radiation was significantly more than control, ionizing radiation, and ketogenic diet alone (*, $P < 0.05$).

in tumor tissue resulting from lipid peroxidation-derived aldehydes.

Ketogenic diets in combination with ionizing radiation decrease immunoreactive PCNA protein in tumor tissue following treatment with fractionated radiation

To further probe possible mechanisms responsible for the inhibition of tumor growth seen when the ketogenic diet was combined with conventional fractionated radiation, tumors harvested at the end of treatment shown in Fig. 2 were analyzed by Western blotting for markers of proliferation (using immunoreactive proliferating nuclear antigen; PCNA) and DNA damage (using immunoreactive phosphorylated histone γ H2AX; Fig. 5). Consistent with the inhibition in tumor growth and prolongation of survival seen in Fig. 2, tumors treated with ketogenic diet + ionizing radiation showed significantly lower levels of immunoreactive PCNA than mice in all other treatment groups (Fig. 5A and B). Phosphorylated histone γ H2AX was significantly increased in tumors treated with radiation; however, mice treated with ketogenic diet + ionizing radiation did not show any further increase in levels of γ H2AX indicating that enhanced radiation response does not seem to be related to increased levels of DNA damage in animals fed a ketogenic diet (Fig. 5C).

Ketones in cell culture media do not significantly enhance radiation response in human lung cancer cells *in vitro*

To mimic ketosis in an *in vitro* system, exponentially growing H292 (Supplementary Fig. S2A) and A549 (Supplementary Fig. S2B) human lung cancer cells were grown in tissue-culture media containing 3 mmol/L β BHB and 1.6 mmol/L AA and 70 mg/dL glucose for 24 hours. These concentrations of ketones and glucose mimic the concentrations and approximate ratios found in the blood of adult humans eating a ketogenic diet (14). Glucose was used at a rate of approximately 35 mg/dL per day depending on the cell concentration and was replenished daily to 70 mg/dL. A549 cells were capable of metabolizing β BHB as shown by a decrease in β BHB levels in the media during 5 days of growth (from 3 to 1.8 mmol/L). The combination of ketones with radiation exposure (0–4 Gy) did not alter clonogenic survival in either H292 or A549 cells, relative to similarly treated cells in standard media (Supplementary Fig. S2A and S2B).

Discussion

The current results show that a ketogenic diet is an effective adjuvant capable of enhancing radio-chemo-therapy responses in xenograft models of human NSCLC. It is also important to note that the ketogenic diet alone in our study did not result in any inhibition of tumor growth, relative to control. This finding is in agreement with 2 studies in orthotopic models of brain cancer (20, 21) where Maurer and colleagues showed no improvement in survival when animals were fed a ketogenic diet as well as Zhou and colleagues who could only show a ketogenic diet-induced

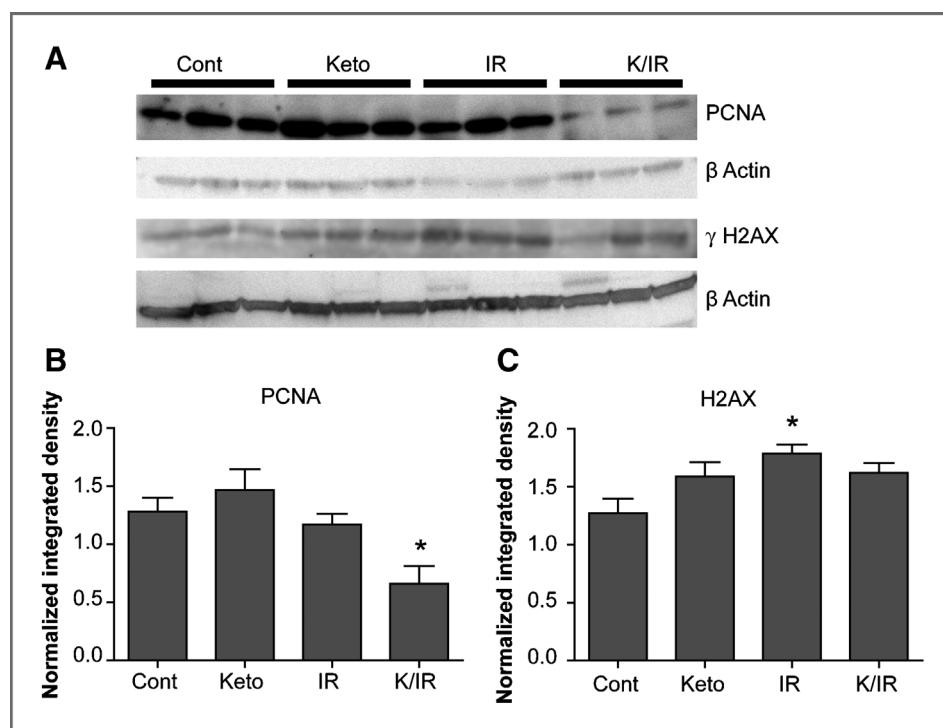


Figure 5. Ketogenic diets combined with fractionated radiation treatment results in decreased immunoreactive PCNA in tumor tissue. Mouse xenograft tumors treated as in Fig. 2 were harvested 24 hours after the final 2 Gy radiation fraction. Tumor protein homogenates harvested from control, ketogenic diet, ionizing radiation, and ketogenic diet + ionizing radiation-treated animals (3 from each group) were separated on denaturing gels, blotted onto membranes, and stained with anti-PCNA or anti- γ H2AX antibodies (A). Quantification of dot blots was done by ImageJ analysis and samples were normalized to the background on each blot. Animals treated with ketogenic diet + ionizing radiation had a significant reduction in PCNA staining, relative to ionizing radiation animals (B). There was not a significant difference in γ H2AX staining when ionizing radiation and ketogenic diet + ionizing radiation were compared, suggesting that ketogenic diets do not increase DNA damage when combined with radiation (C). Error bars represent \pm 1SEM. One way ANOVA with Newman–Keuls Multiple Comparison Test were used to determine which groups were significantly different from control (*, $P < 0.05$).

tumor growth inhibition when the diet was combined with caloric restriction. However our findings in lung cancer xenografts differ from other investigations in prostate, gastric, and brain cancer models that show a positive therapeutic advantage using ketogenic diet as a monotherapy (22–24). These differences could be due to the different types of tumor and animal models used or the different fatty acid compositions of the ketogenic diets and control diets that were used. Our model uses a soy-based fatty acid composition which is relatively high in oleic (18:1), linoleic (18:2), and linolenic (18:3) acids with standard mouse chow as the control. Because unsaturated fatty acids are substrates for lipid peroxidation that may govern the formation of aldehydes such as 4HNE, this fatty acid composition may be influencing our results. Freedland and colleagues used a high fat, moderate high carbohydrate "Western" diet as the control diet in a prostate cancer model (23), whereas Otto and colleagues used a ketogenic diet supplemented with omega 3 fatty acids and medium-chain triglycerides (22). In addition, Stafford and colleagues used a syngeneic intracranial mouse model of glioma showing the potential role of tumor microenvironment in the biologic effects of ketogenic diets (24).

The current study also showed that ketogenic diets enhanced the responses of H292 and A549 NSCLC xenografts using 2 different radiation dosing schemes. In the conventional fractionation dosing scheme (61.2 Gy in 34 fractions with 1.8 Gy/fraction), significant increases in animal survival were achieved in the ketogenic diet + ionizing radiation group when compared with the ionizing radiation alone treatment group (Fig. 2). In a recent clinical trial, studying the effects of a ketogenic diet on the quality of life in 16 patients with advanced cancer, it was concluded that the ketogenic diet was suitable for patients with advanced cancer and resulted in no severe side effects (25). However, it was also noted that 4 of the 16 patients stopped the diet before the end of a 7-week treatment time (25).

To shorten the time required to be maintained on a ketogenic diet, xenograft responses to hypofractionated radiotherapy were determined. Clinically, hypofractionation allows for both increased patient convenience and reduced costs without sacrificing efficacy and is frequently used to treat early-stage lung cancers (19, 26–28). Ketogenic diet combined with hypofractionated ionizing radiation significantly slowed tumor growth rate and prolonged survival, when compared with radiation alone, in

both the H292 and A549 lung cancer xenograft models (Fig. 3A and B). This finding in lung cancer is similar to a recent finding using a ketogenic diet with hypofractionated radiation (2×4 Gy fractions) to treat transplantable mouse gliomas (29).

Elevated ketone levels have been implicated in the generation of cellular metabolic oxidative stress. Jain and colleagues found elevated indices of lipid peroxidation in cultured human endothelial cells treated with AA (30). Stadler and colleagues showed an increase in protein oxidation and lipid peroxidation through a free radical-dependent mechanism in rat livers of animals subjected to a model of acetone induced ketosis (31). Chronic exposure to β BHB was also shown to increase ROS production in cardiomyocytes (32). Finally, Milder and colleagues showed an increase in H_2O_2 production and lipid peroxidation in the hippocampus of rats fed a ketogenic diet (33). An excellent review addressing the effect of ketogenic diet on redox biology in neuronal tissues was also recently published (34).

It has been hypothesized that cancer cells, relative to normal cells, exist in a condition of chronic metabolic oxidative stress mediated by increased steady-state levels of O_2^- and H_2O_2 , with a major site of prooxidant production being mitochondrial electron transport chain complexes. This increased level of ROS in cancer cells may be compensated for by increases in glucose metabolism though the pentose phosphate pathway resulting in the generation of NADPH to be used as a cofactor in hydroperoxide metabolism (3). Because ketogenic diets force cells to rely on mitochondrial oxidative metabolism for energy production while limiting glucose availability, it would be expected that a ketotic state would exacerbate metabolic oxidative stress in cancer cells, relative to normal cells. Thus, a ketogenic diet may selectively enhance radio-chemo-responses in cancer cells when combined with standard cancer therapeutics through a mechanism involving oxidative stress.

Consistent with this hypothesis, the current studies also showed that tumors from mice treated with ketogenic diet and radiation showed significantly increased 4HNE-modified proteins (Fig. 4). This observation supports the hypothesis that ketogenic diet-induced enhancement of radiation response *in vivo* may involve increases in lipid peroxidation and protein damage that could contribute to growth inhibition as assayed by lower levels of immunoreactive PCNA (Fig. 5). The accumulation of 4HNE-modified proteins in tumors treated with the ketogenic diet combined with radiation are also consistent with previous results in breast cancer cells showing that exposure to a mixture of conjugated linoleic acid isomers could selectively inhibit breast cancer cell proliferation by increasing levels of 4HNE (35).

Interestingly, H292 and A549 cells exposed *in vitro* to ketones and ionizing radiation doses ranging from 0.5 Gy to 4 Gy did not show enhanced radiation responses. Previous research has also showed that radiation responses *in vitro* do not necessarily correlate with radiation responses *in vivo* (36). Radiation response *in vivo* may be influenced

by a variety of factors including oxygen effects, the presence of an immune system, antioxidant levels, DNA repair and the extracellular environment that are not adequately modeled *in vitro*. As an example, the *in vitro* clonogenic survival assays with ketones were conducted in 21% oxygen which is significantly higher than the partial pressure of oxygen in solid tumors of animals fed ketogenic diets (37). Furthermore, therapeutic radiation doses also initiate a cascade of signaling events involving cytokines and oxidant-producing pathways that further induce activation of the immune system and inflammation that contributes to tissue damage and radiation responses that is not adequately modeled *in vitro* (38). Our data suggest that when studying the effects of ketogenic diets on radiation responses that *in vivo* model systems may be more appropriate than *in vitro* model systems.

The current study supports the conclusion that ketogenic diets are well tolerated by tumor-bearing animals receiving concurrent radio-chemo-therapy. These results also support the conclusion that feeding a ketogenic diet could serve as an easily implemented adjuvant for improving responses to radio-chemo-therapies in the treatment of lung cancer. Finally, these data also support the hypothesis that a ketogenic diet enhances radiation responses in lung cancer xenografts by a mechanism involving oxidative stress and inhibition of cancer cell proliferation *in vivo*.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Acknowledgments

The authors thank Drs. Andrean Simons-Burnett, Daniel Berg, Thor Halldanarson, Zita Sibenaller, James Jacobus, Alf Siochi as well as Sam Rodman, Tyler Ronnfeldt, Amanda Kalen, Ryan Beckenbaugh, Kellie Bodeker, Gareth Smith, Mindi TenNapel, and Starlette Sharpe for their technical assistance and helpful discussions.

Grant Support

The work was supported by the NIH (CA139182, UL1RR024979, and CA133114), Radiological Society of North America (RR1020), The Carver Trust Research Program of Excellence in Redox Biology and Medicine, and a gift from Marie Foster, Nellie K. Spitz, and IBM. B.G. Allen is a B. Leonard Holman Pathway medical resident supported by The Department of Radiation Oncology at The University of Iowa Hospitals and Clinics.

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Received January 25, 2012; revised May 3, 2013; accepted May 27, 2013; published OnlineFirst June 6, 2013.

References

- de Geus-Oei LF, van Krieken JH, Aliredjo RP, Krabbe PF, Frieling C, Verhagen AF, et al. Biological correlates of FDG uptake in non-small-cell lung cancer. *Lung Cancer* 2007;55:79–87.
- Porporato PE, Dhup S, Dadhich RK, Copetti T, Sonveaux P. Anticancer targets in the glycolytic metabolism of tumors: a comprehensive review. *Front Pharmacol* 2011;2:49.
- Aykin-Burns N, Ahmad IM, Zhu Y, Oberley LW, Spitz DR. Increased levels of superoxide and H₂O₂ mediate the differential susceptibility of cancer cells versus normal cells to glucose deprivation. *Biochem J* 2009;418:29–37.
- Bize IB, Oberley LW, Morris HP. Superoxide dismutase and superoxide radical in Morris hepatomas. *Cancer Res* 1980;40:3686–93.
- Springer EL. Comparative study of the cytoplasmic organelles of epithelial cell lines derived from human carcinomas and nonmalignant tissues. *Cancer Res* 1980;40:803–17.
- Parrella P, Xiao Y, Fliss M, Sanchez-Cespedes M, Mazzarelli P, Rinaldi M, et al. Detection of mitochondrial DNA mutations in primary breast cancer and fine-needle aspirates. *Cancer Res* 2001;61:7623–6.
- Wang X, Perez E, Liu R, Yan LJ, Mallet RT, Yang SH. Pyruvate protects mitochondria from oxidative stress in human neuroblastoma SK-N-SH cells. *Brain Res* 2007;1132:1–9.
- Fath MA, Diers AR, Aykin-Burns N, Simons AL, Hua L, Spitz DR. Mitochondrial electron transport chain blockers enhance 2-deoxy-D-glucose induced oxidative stress and cell killing in human colon carcinoma cells. *Cancer Biol Ther* 2009;8:1228–36.
- McPherson PA, McEneny J. The biochemistry of ketogenesis and its role in weight management, neurological disease, and oxidative stress. *J Physiol Biochem* 2012;68:141–51.
- Westman EC, Yancy WS Jr, Mavropoulos JC, Marquart M, McDuffie JR. The effect of a low-carbohydrate, ketogenic diet versus a low-glycemic index diet on glycemic control in type 2 diabetes mellitus. *Nutr Metab (Lond)* 2008;5:36.
- Yancy WS Jr, Westman EC, McDuffie JR, Grambow SC, Jeffreys AS, Bolton J, et al. A randomized trial of a low-carbohydrate diet vs orlistat plus a low-fat diet for weight loss. *Arch Intern Med* 2010;170:136–45.
- Haddad F, Baldwin KM, Morris GS. Dietary effects on cardiac metabolic properties in rodents. *J Mol Cell Cardiol* 1990;22:353–9.
- Brito SR, Moura MA, Kawashita NH, Brito MN, Kettellhut IC, Migliorini RH. Glucose uptake and glycolytic flux in adipose tissue from rats adapted to a high-protein, carbohydrate-free diet. *Metabolism* 2001;50:1208–12.
- Phinney SD, Bistrian BR, Wolfe RR, Blackburn GL. The human metabolic response to chronic ketosis without caloric restriction: physical and biochemical adaptation. *Metabolism* 1983;32:757–68.
- Fath MA, Ahmad IM, Smith CJ, Spence J, Spitz DR. Enhancement of carboplatin-mediated lung cancer cell killing by simultaneous disruption of glutathione and thioredoxin metabolism. *Clin Cancer Res* 2011;17:6206–17.
- Seyfried TN, Sanderson TM, El-Abbad MM, McGowan R, Mukherjee P. Role of glucose and ketone bodies in the metabolic control of experimental brain cancer. *Br J Cancer* 2003;89:1375–82.
- Fath MA, Simons AL, Erickson J, Anderson ME, Spitz DR. Enhancement of cancer therapy using ketogenic diet. In: Spitz DR, Dornfeld KJ, Krishnan K, Guis D, editors. *Oxidative stress in cancer biology and therapy*. Humana Press; 2012; p. 47–58.
- Cohn JA, Tsai L, Friguet B, Szweda LI. Chemical characterization of a protein-4-hydroxy-2-nonenal cross-link: immunochemical detection in mitochondria exposed to oxidative stress. *Arch Biochem Biophys* 1996;328:158–64.
- Fakiris AJ, McGarry RC, Yiannoutsos CT, Papiez L, Williams M, Henderson MA, et al. Stereotactic body radiation therapy for early-stage non-small-cell lung carcinoma: four-year results of a prospective phase II study. *Int J Radiat Oncol Biol Phys* 2009;75:677–82.
- Zhou W, Mukherjee P, Kiebish MA, Markis WT, Mantis JG, Seyfried TN. The calorically restricted ketogenic diet, an effective alternative therapy for malignant brain cancer. *Nutr Metab* 2007;4:5.
- Maurer GD, Brucker DP, Bahi O, Harter PN, Hattingen E, Walenta S, et al. Differential utilization of ketone bodies by neurons and glioma cell lines: a rationale for ketogenic diet as experimental glioma therapy. *BMC Cancer* 2011;11:315.
- Otto C, Kaemmerer U, Illert B, Muehling B, Pfetzer N, Wittig R, et al. Growth of human gastric cancer cells in nude mice is delayed by a ketogenic diet supplemented with omega-3 fatty acids and medium-chain triglycerides. *BMC Cancer* 2008;8:122.
- Freedland SJ, Mavropoulos J, Wang A, Darshan M, Demark-Wahnefried W, Aronson WJ, et al. Carbohydrate restriction, prostate cancer growth, and the insulin-like growth factor axis. *Prostate* 2008;68:11–9.
- Stafford P, Abdelwahab MG, Kim do Y, Preul MC, Rho JM, Scheck AC. The ketogenic diet reverses gene expression patterns and reduces reactive oxygen species levels when used as an adjuvant therapy for glioma. *Nutr Metab* 2010;7:74.
- Schmidt M, Pfetzer N, Schwab M, Strauss I, Kammerer U. Effects of a ketogenic diet on the quality of life in 16 patients with advanced cancer: a pilot trial. *Nutr Metab* 2011;8:54.
- Timmerman RD, Story M. Stereotactic body radiation therapy: a treatment in need of basic biological research. *Cancer J* 2006;12:19–20.
- Onishi H, Araki T, Shirato H, Nagata Y, Hiraoka M, Gomi K, et al. Stereotactic hypofractionated high-dose irradiation for stage I non-small-cell lung carcinoma: clinical outcomes in 245 subjects in a Japanese multiinstitutional study. *Cancer* 2004;101:1623–31.
- Cheung PC, Yeung LT, Basrur V, Ung YC, Balogh J, Danjoux CE. Accelerated hypofractionation for early-stage non-small-cell lung cancer. *Int J Radiat Oncol Biol Phys* 2002;54:1014–23.
- Abdelwahab MG, Fenton KE, Preul MC, Rho JM, Lynch A, Stafford P, et al. The ketogenic diet is an effective adjuvant to radiation therapy for the treatment of malignant glioma. *PLoS ONE* 2012;7:e36197.
- Jain SK, Kannan K, Lim G. Ketosis (acetooacetate) can generate oxygen radicals and cause increased lipid peroxidation and growth inhibition in human endothelial cells. *Free Radic Biol Med* 1998;25:1083–8.
- Stadler K, Bonini MG, Dallas S, Duma D, Mason RP, Kadiiska MB. Direct evidence of iNOS-mediated *in vivo* free radical production and protein oxidation in acetone-induced ketosis. *Am J Physiol Endocrinol Metab* 2008;295:E456–62.
- Pelletier A, Coderre L. Ketone bodies alter dinitrophenol-induced glucose uptake through AMPK inhibition and oxidative stress generation in adult cardiomyocytes. *Am J Physiol Endocrinol Metab* 2007;292:E1325–32.
- Milder JB, Liang LP, Patel M. Acute oxidative stress and systemic Nrf2 activation by the ketogenic diet. *Neurobiol Dis* 2010;40:238–44.
- Milder JB, Patel M. Modulation of oxidative stress and mitochondrial function by the ketogenic diet. *Epilepsy Res* 2012;100:295–303.
- Albright CD, Klem E, Shah AA, Gallagher P. Breast cancer cell-targeted oxidative stress: enhancement of cancer cell uptake of conjugated linoleic acid, activation of p53, and inhibition of proliferation. *Exp Mol Pathol* 2005;79:118–25.
- Malaise EP, Fertil B, Chavaudra N, Guichard M. Distribution of radiation sensitivities for human tumor cells of specific histological types: comparison of *in vitro* to *in vivo* data. *Int J Radiat Oncol Biol Phys* 1986;12:617–24.
- Hockel M, Vaupel P. Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. *J Natl Cancer Inst* 2001;93:266–76.
- Schaeue D, McBride WH. Links between innate immunity and normal tissue radiobiology. *Radiat Res* 2010;173:406–17.