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Effect of weight gain and weight loss on *in vivo* colonocyte proliferation rate in people with obesity

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Abstract

Objective—To evaluate the effects of diet-induced changes in energy balance and body weight on *in vivo* colonocyte fractional proliferation rates (FPR) in people with obesity.

Methods—*In vivo* colonocyte FPR was assessed in 31 men and women with obesity (BMI: 35.4±4.0 kg/m^2, age: 52.6±8.9 years) before and after diet-induced weight loss, weight gain, or weight maintenance. Subjects ingested aliquots of ^{2}H$_2$O (heavy water) daily for 4-7 days, followed by flexible sigmoidoscopy with colon biopsies to assess the incorporation of ^{2}H into the DNA of dividing colonocytes.

Results—Colonocyte FPR averaged 12.7±3.8 % per day, and correlated directly with adipose tissue (IAAT) volume (r=0.364, *P*=0.044). Colonocyte FPR decreased in the weight loss group, did not change in the weight maintenance group, and increased in the weight gain group. The change in colonocyte FPR correlated directly with the percent change in body weight (r=0.409, *P*=0.028) and IAAT volume (r=0.598, *P*=0.001).

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Conclusions—A high-calorie diet and weight gain increase, whereas a low-calorie diet and weight loss decrease, *in vivo* colonocyte proliferation rate in people with obesity. These results suggest that changes in energy balance influence the risk of developing colon cancer in people with obesity by regulating colonic mucosal growth rates.

Keywords

colon cancer; isotope tracers; MIDA

INTRODUCTION

Colorectal cancer is the third most common cancer worldwide and accounts for 5% – 10% of the incidence of all cancers and cancer-related deaths (1). Data from case-control and cohort studies indicate that increased body mass index (BMI) is associated with a dose-dependent increase in colorectal cancer incidence (2-5). Moreover, increased intra-abdominal adipose tissue (IAAT) is more closely associated with markers of colonic carcinogenesis and colon cancer risk than BMI *per se* (6-8). Although these data underscore the potential importance of excess adiposity in the pathogenesis of colon cancer, the mechanism(s) responsible for this link in people is not clear.

The development of colon cancer is a multi-step process beginning with an increase in colonocyte proliferation rate that can increase the risk of cancer by increasing the likelihood of mutations caused by DNA replication errors or large scale chromosomal abnormalities, thereby generating neoplastic polyps that can undergo malignant transformation to colon cancer (9). Therefore, the rate of epithelial cell proliferation is an early indicator of colorectal cancer risk (10,11). S phase DNA replication during mitosis is likely directly involved in the pathogenesis of colon carcinogenesis (12), by decreasing the time available for DNA repair, which allows DNA damage to become a permanent mutation, and by generating DNA replication errors (13).

Data from studies conducted in animal models have shown weight gain and obesity increase colonocyte proliferation rates and induce colon tumorigenesis (14,15), whereas calorie restriction and weight loss decrease colonocyte proliferation rates and the formation of colonic neoplasms (16,17). There is also evidence that obesity is associated with increased colonocyte proliferation rates in people, as markers of increased colorectal epithelial cell mitosis have been found in people with extreme obesity compared with those who have normal weight (18). However, the effect of weight loss on colonocyte proliferation in people with obesity is unclear because of conflicting results from different studies, which found weight loss reduced *ex vivo* [³H]thymidine incorporation in colonic crypt cells (a biomarker of cell proliferation) (19), or increased immunohistochemical markers of mitosis in colonic epithelial cells (18). The reason for the inconsistency between these studies is not known, but could be due to differences in the methods used to assess colonocyte proliferation, which provided *ex vivo* rather than direct *in vivo*, assessments of epithelial cell proliferation.

The major aims of the present study were to: 1) determine whether the assessment of *in vivo* colonocyte proliferation rates, measured by using a recently developed stable isotope tracer method that labels the DNA of dividing cells *in vivo* (20,21), is consistent with the results

obtained from a standard *ex vivo* staining method of cell proliferation; and 2) determine the effect of diet-induced weight gain and weight loss on *in vivo* colonocyte proliferation rates in people with obesity.

METHODS

Subjects

Thirty-one subjects with obesity (28 women and 3 men; BMI: 35.4 ± 4.0 kg/m^2, age: 52.6 ± 8.9 years) participated in this study. The assessment of colonocyte proliferation in these subjects was made during their participation in other studies that involved diet-induced weight loss (n = 17) (22,23), diet-induced weight gain (n = 7) (24), or no change in body weight (n = 7) (22). Two subjects participating in the weight loss study withdrew before completion, so that only baseline measurements are available for them. All subjects completed a comprehensive medical evaluation at baseline, which included a detailed history and physical examination, routine blood tests, and a 2-hour oral glucose tolerance test. Participants who had diabetes, gastrointestinal disease, previous gastrointestinal surgery, consumed 20 g/d of alcohol, or smoked cigarettes were excluded. Subjects provided their written informed consent before participating in this study, which was approved by the Washington University Human Research Protection Office.

Experimental procedures

Body composition—Whole-body fat and fat-free masses were determined by using dual-energy X-ray absorptiometry (Lunar iDXA, GE Healthcare; Madison, WI), IAAT volume was quantified by using magnetic resonance imaging (Magnetom, Siemens Medical Solutions; Malvern, PA) and Analyze 7.0 software (Biomedical Imaging Resource, Mayo Clinic; Rochester, MN), and intrahepatic triglyceride (IHTG) content was quantified by using magnetic resonance spectroscopy (Siemens and Mayo Clinic) (22-24).

In vivo DNA labeling procedure—Subjects were admitted to the Washington University School of Medicine Clinical Research Unit (CRU) in the morning, after they fasted for ~12 h overnight at home. Starting at ~0900 h, they ingested 50 ml of 70% ^2H_2O (Isotec, Sigma-Aldrich; St. Louis, MO) every 3 hours for 24 hours (total of 8 doses; loading phase). Blood samples were obtained at t = 0 (before tracer administration) and at 6, 12, 18, 21, and 24 h to determine body water ^2H enrichment. Subjects were then discharged from the CRU and additional individual 50 ml doses of 70% ^2H_2O, dispensed in sterile plastic vials, were provided to them for consumption at home for the next 3-6 days (1 dose every 12 hours; maintenance phase). Saliva samples were collected at home before ingesting each ^2H_2O dose, to determine body water ^2H enrichment. Saliva samples were collected in sterile salivettes (Sarstedt Salivette Cotton Swab, Fisher Scientific Company; Hanover Park, IL) and placed in a freezable bag (PackIt Freezable Bag, PackIt; Westlake Village, CA) until subjects returned to the CRU for sigmoidoscopy, ~12 h after the final ^2H_2O dose. The maintenance phase varied from 3-6 days to accommodate subject availability and procedure scheduling. The period of dosing was 4.6 ± 0.8 days before and 4.4 ± 0.7 days after the intervention.

Flexible sigmoidoscopy—Flexible sigmoidoscopy with sigmoid colon biopsies was performed in the morning after the last dose of ^2H_2O was consumed in the evening at home. After subjects fasted for ~12 h overnight at home, they were admitted as outpatients to the CRU. Flexible sigmoidoscopy was performed by a single gastroenterologist (S. Sullivan), without anesthesia, and 8-10 mucosal biopsies were obtained from the sigmoid colon (25-35 cm from the anal verge) by using standard procedures. The location was noted and repeat biopsies after the intervention were taken at the same distance from the anal verge. Biopsy specimens were rinsed immediately in 0.9% ice-cold saline, and blotted dry to remove any blood and fecal material; two specimens were immediately fixed in 10% formalin and subsequently embedded in OTC compound for histological analysis, whereas the remaining biopsies were immediately frozen in liquid nitrogen and stored at -80°C until further processing.

Diet intervention—After baseline procedures were completed, subjects participated in study protocols that involved moderate (~5%) diet-induced weight gain (n = 7), variable (5% – 15%) diet-induced weight loss (n = 17; 15 completers), or maintenance of body weight for ~6 months (n = 7), as previously described (22-24). At the end of the weight gain and weight loss diet interventions, a weight-maintenance diet was prescribed and subjects were kept weight-stable (<2% change in body weight) for 2-4 weeks before repeating the same study procedures conducted at baseline.

Evaluation of colonocyte proliferation

In vivo colonocyte proliferation rate—This procedure is based on the incorporation of deuterium (^2H) from ^2H_2O (heavy water) into the deoxyribose (dR) moiety of purine deoxyribonucleotides in DNA of dividing cells (20,21). This approach involves rapidly increasing the deuterium content of body water over 24 hours and then maintaining a constant enrichment over the next 4-7 days. Any colonocyte that goes through the S-phase of the cell cycle during this period will contain deuterium in the dR moiety of deoxyribonucleotides in its DNA (20). Accordingly, when a cell undergoes cell division, 1/2 of the DNA in the two daughter cells will be labeled with ^2H after one round of proliferation, 3/4 after two rounds, 7/8 after three rounds, etc. Therefore, an assessment of the cells with labeled DNA provides a measure of the fraction (f) of cells in the biopsy population that divided during the period of ^2H_2O exposure. The maximum f value observed in any of our subjects, either before or after the interventions, was 57%, indicating that all colon biopsy samples were obtained during the rise to plateau and not near or at plateau (i.e. all cells being labeled). Initially after starting a ^2H_2O labeling protocol, most of the cells that slough from the colonic crypts into the intestinal lumen will likely be unlabeled, whereas all dividing cells in the crypt will contain label. During the next several days, both labeled and unlabeled cells will slough off and be replaced by labeled cells that divided during the period of ^2H_2O exposure. The fractional replacement rate of old cells by newly divided cells is the fractional proliferation rate (FPR), expressed as % per day.

The FPR of colonocytes was calculated by using the precursor – product relationship. The maximal theoretical isotopic enrichment of completely turned-over cells at the ^2H_2O

exposure measured in each subject was used as the denominator for calculating the fraction of newly divided colonocytes (f) (21):

- fraction of newly divided cells (f) = dR enrichment (EM_1) in sample cells / dR enrichment (EM_1) in fully turned-over cells

- fractional proliferation rate constant (FPR) = [– ln (1 – f)] / t

where t is the period of heavy water dosing, and EM_1 is the excess M+1 mass isotopomer abundance over natural abundance values. Maximal EM_1 enrichment of dR in fully-turned-over cells was calculated from ^2H_2O exposure by using combinatorial principles (mass isotopomer distribution analysis, MIDA) (21). Colonocyte half-life was calculated as ln 2/FPR = 0.693/FPR and residence time was calculated as 1/FPR.

To determine *in vivo* colonocyte proliferation rate, paired frozen biopsies from each subject before and after the intervention were thawed and incubated in 5 mM EDTA in PBS for 30 min at 37 °C. Loosened epithelial cells were recovered by vortexing the segment, aspirating repeatedly through a pipette and centrifugation. Cells were lysed with protease treatment, and DNA was recovered by using a commercial recovery column (DNEasy, Qiagen; Germantown, MD). The purified DNA was hydrolyzed to free nucleosides by using nuclease and phosphatase enzyme digestion. Pentafluorobenzylhydroxylamine derivatives of the dR portions of the nucleosides were prepared for GCMS analysis. Mass spectra of dR derivatives identified newly synthesized dR molecules based on incorporation of deuterium in place of unlabeled hydrogen.

Ex vivo colonocyte proliferation rate—The Ki-67 immunohistochemical staining method of estimating cell proliferation was evaluated in 16 samples. This procedure evaluates the percentage of crypt cells that stain positive for Ki-67 protein, which is a specific nuclear marker of cell proliferation. Ki-67 staining was performed in OCT-embedded colon tissue to obtain a commonly used metric of cell proliferation. Slides were visualized at ×20 magnification on a Zeiss Axioskop 2 MOT microscope (Carl Zeiss Microscopy GmbH; Jena, Germany) equipped with a Nuance FX Multispectral Imaging System camera (Cambridge Research and Instrumentation; Woburn, MA). To obtain a more robust estimate of Ki-67 labeling, four representative images were taken for each tissue sample by using Nuance 2.10 software, and positive and negative crypt nuclei were counted by using inForm 1.4 software (Caliper Life Sciences; Hopkinton, MA); on average, 3239 ± 787 cells were counted for each tissue sample.

Statistical analysis

All data sets were tested for normality according to Shapiro-Wilks, and parametric (paired t-test, Pearson correlation) or non-parametric (Wilcoxon sign ranked test, Spearman correlation) procedures were used to analyze the data, as needed. Differences between weight loss and weight gain groups were analyzed by analysis of variance after adjusting for baseline values and duration of the diet intervention. All statistical analyses were performed with SPSS version 23 (IBM SPSS, Chicago, IL). Summary results are reported as means ± SD, unless otherwise indicated. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Correlation between *in vivo* and *ex vivo* assessments of colonocyte proliferation rates

In vivo colonocyte FPR averaged 12.7 ± 3.8 % per day, indicating that ~13% of the colonic epithelial cells isolated were replaced or renewed every day. These results indicate a mean residence time of ~7.9 days and a half-life of ~5.5 days. The corresponding *ex vivo* Ki-67 labeling was 69.9 ± 10.8 %, indicating that ~70% of colonocytes were in an active phase of the cell cycle at the time of biopsy. The *in vivo* colonocyte FPR correlated directly with Ki-67 labeling (r = 0.567, $P = 0.022$; Figure 1).

Relationships between baseline colonocyte FPR and body composition

At baseline (n = 31), *in vivo* colonocyte FPR correlated positively with IAAT volume (r = 0.364, $P = 0.044$; Figure 2) but not with other anthropometric or body composition variables (body weight, BMI, % body fat, whole-body fat mass in kg, and IHTG content).

Effects of weight loss and weight gain on colonocyte FPR

Mean body weight decreased by 9.8 ± 3.5 % (range 5% – 15%) in subjects assigned to weight loss (n = 15), increased by 5.0 ± 0.8 % in subjects assigned to weight gain (n = 7), and did not change (-0.1 ± 1.8 %) in subjects assigned to the control intervention (n = 7). Mean colonocyte FPR decreased in the weight loss group, did not change in the control group, and increased in the weight gain group (Figure 3). The change in colonocyte FPR after weight loss (-3.0 ± 1.3 % per day) was significantly different ($P = 0.045$) from the change in colonocyte FPR after weight gain (2.5 ± 1.9 % per day). The intervention-induced changes in colonocyte FPR correlated positively with the percent changes in body weight (r = 0.409, $P = 0.028$; Figure 4 *top*) and IAAT volume (r = 0.598, $P = 0.001$; Figure 4 *bottom*). In addition, mean fasting plasma insulin concentration decreased after weight loss (by 32 ± 13%), increased after weight gain (by 53 ± 21%) and did not change after weight maintenance (3 ± 18%); the changes after weight loss and weight gain were significantly different from each other ($P = 0.005$).

DISCUSSION

Colon cancer represents the end result of a progression of changes in colonic epithelia from normal mucosa to increased colonocyte proliferation, formation of benign neoplasms and finally malignant transformation. An increase in colonocyte proliferation is considered an early marker of colon cancer risk, and is likely directly involved in the pathogenesis of carcinogenesis (10,11). In this study, we evaluated the effect of diet-induced weight gain and weight loss on *in vivo* colonocyte proliferation rates in people with obesity, by using a recently developed stable isotope tracer method that uses ^2H_2O to endogenously label the DNA of dividing cells (20,21). We found that about 13% of colonocytes were renewed daily, and that diet-induced changes in body weight influenced colonocyte FPR. Weight gain and weight loss caused an increase and a decrease, respectively, in colonocyte FPR and the percent change in body weight correlated directly with the change in colonocyte FPR. These results demonstrate an important link between energy balance and colon cancer risk, mediated by the rate of colonocyte proliferation.

The ^2H_2O labeling method that we used to assess *in vivo* colonocyte proliferation rates has been used previously in animal models (25,26), but we are not aware of any studies that have used this approach in people. Therefore, we evaluated the reliability of the ^2H_2O method by comparing the results obtained with this method to those obtained by using a standard *ex vivo* Ki-67 labelling technique in the same subjects. The *in vivo* colonocyte FPR correlated directly with the corresponding *ex vivo* estimate across a wide range of values. About 70% of colonocytes stained positive for Ki-67, which represents the fraction of cells "in cycle". This value was ~6-fold greater than the percentage of cells that had incorporated the ^2H_2O tracer into DNA each day during the previous 4-7 days. DNA replication, a direct marker of cell proliferation, occurs in the S phase of the cell cycle, whereas Ki-67 protein is present during the interphase and all subsequent active phases of the cell cycle (G1, S, G2, and mitosis) and is only absent in resting cells (G0). The duration of the S phase of normal human colonic mucosa is ~11 hours and the total cell cycle duration involving all phases is ~73 hours (27). Furthermore, only 15% of normal human sigmoid colon epithelial cells are in the S phase at any given time (28). Accordingly, these data suggest *ex vivo* Ki-67 labeling overestimates the true *in vivo* daily colonocyte proliferation rate. The quantitative estimates of colonocyte FPR in our subjects indicate that colonocytes have a residence time of ~7.9 days, or a half-life of ~5.5 days, which are within the range of previous indirect estimates of human colorectal epithelial cell turnover rates (1-8 days; mean of ~4 days) (29).

Our data support the notion that changes in energy balance and body weight affect the risk of developing colon cancer. However, our study is limited because it provides only a short-term assessment of dietary manipulation and only evaluates a single risk factor for colon cancer, not colon cancer itself. Conclusions about weight change and colon cancer from long-term retrospective and prospective epidemiological studies are unclear because of conflicting results among studies, which have found the incidence of colon cancer increases or does not change with weight gain, and decreases or does not change with weight loss (5,30-33). The reason for the different outcomes among studies is unclear, but could be related to differences in age of the participants, accuracy of body weight recall in retrospective studies, the ability to distinguish between intentional and unintentional weight loss, the maintenance of weight loss, and inadequate duration of follow-up. Studies conducted in animal models are consistent with our findings in people and demonstrate that a high-calorie diet and increased body weight increase (14,15), whereas calorie restriction and blunted weight gain decrease (16,34,35), the occurrence and progression of colon tumors.

In our subjects, baseline IAAT volume and diet-induced changes in IAAT volume were more strongly correlated with colonic FPR than BMI or total body fat mass. This observation is consistent with the relationship between abdominal obesity and colon cancer risk reported in case-control and cohort studies (6-8). Although our study is not able to determine the cellular mechanisms responsible for the effect of weight gain and weight loss on colonic FPR, data from studies conducted in cancer patients, animal models, and cell systems suggest insulin resistance and both systemic and intestinal inflammation are likely involved in the pathogenesis of obesity-related colon cancer (36,37). Insulin resistance in obesity is associated with an increase in both plasma insulin and insulin-like growth factor-1 concentrations and with chronic low-grade inflammation, which have been shown to

stimulate colonocyte proliferation and are thought to contribute to increased risk of developing colorectal neoplasia (36,37). Accordingly, calorie restriction and weight loss could decrease colon cancer risk by attenuating the metabolic and inflammatory abnormalities associated with obesity (38).

In summary, this study demonstrates that colonocyte proliferation rate, which is a marker of colon cancer risk (10,11), can be measured *in vivo* in people by determining the incorporation of deuterium from ingested ^2H_2O tracer into the DNA of colonic epithelial cells. By using this approach, we found that diet-induced weight gain and weight loss cause a corresponding increase and decrease in colonocyte FPR. These data indicate that colonic epithelia are sensitive to changes in energy balance and body weight, and underscore the potential importance of diet therapy in mitigating colon cancer risk in people with obesity. Additional studies are needed to further evaluate the long-term effects of dietary manipulations, including altering total energy content as well as macronutrient and micronutrient composition, on colonocyte proliferation. Furthermore, the assessment of individual colonocyte FPR *in vivo* in response to specific dietary manipulations provides an opportunity to explore the potential of personalized nutritional therapy to reduce colon cancer risk.

Acknowledgments

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STUDY IMPORTANCE QUESTIONS

What is already known about this subject?

- Colon cancer is the result of a progression of changes in colonic epithelia from normal mucosa to increased colonocyte proliferation, formation of benign neoplasms and finally malignant transformation.

- The rate of colonic epithelial cell proliferation is considered an early indicator of colon cancer risk.

- Obesity, particularly abdominal obesity, is an important risk factor for colon cancer.

What does your study add?

- Established for the first time the *in vivo* turnover rate of colonocytes in free-living human subjects.

- Demonstrated a direct correlation between intra-abdominal adipose tissue volume and colonocyte proliferation rate.

- Demonstrated that weight gain and weight loss cause an increase and a decrease, respectively, in *in vivo* colonocyte proliferation rates in people with obesity.
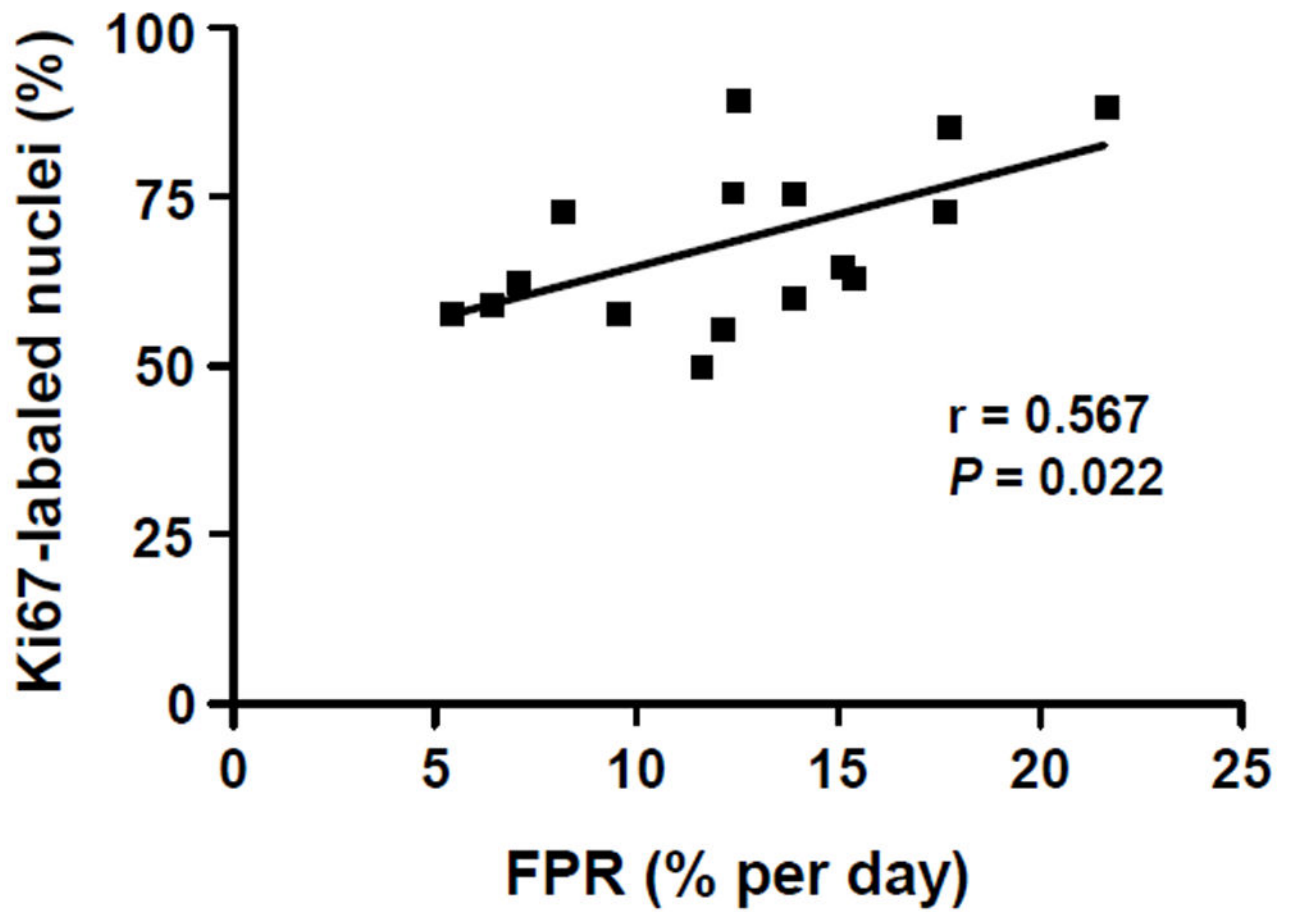
Figure 1.
Relationship between *in vivo* colonocyte fractional proliferation rate (FPR) and *ex vivo* Ki-67 labeling of sigmoid colonic mucosal cells in 16 samples from people with obesity.
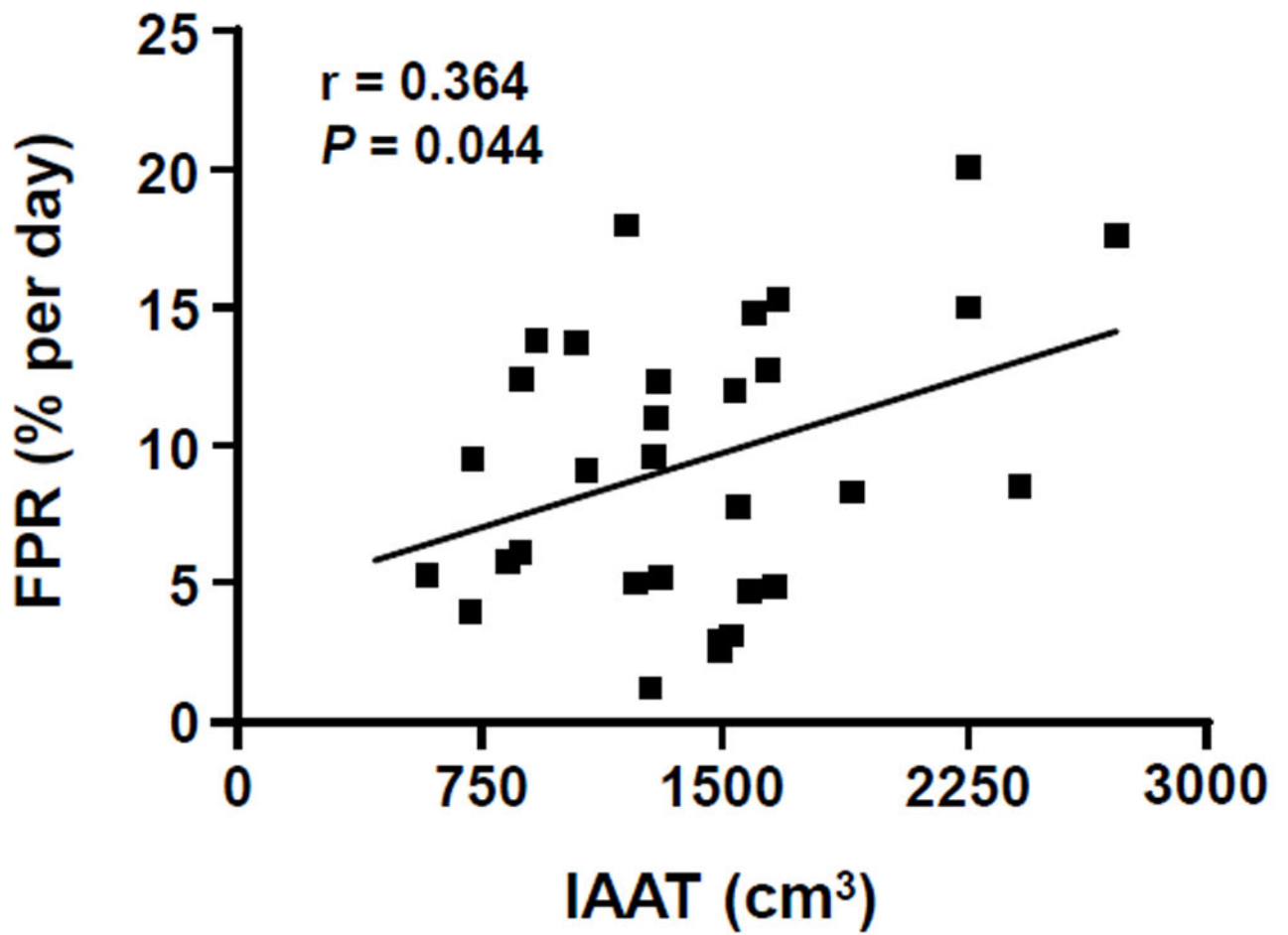
Figure 2.
Relationship between *in vivo* colonocyte fractional proliferation rate (FPR) and intra-abdominal adipose tissue (IAAT) volume at baseline in people with obesity (n = 31).
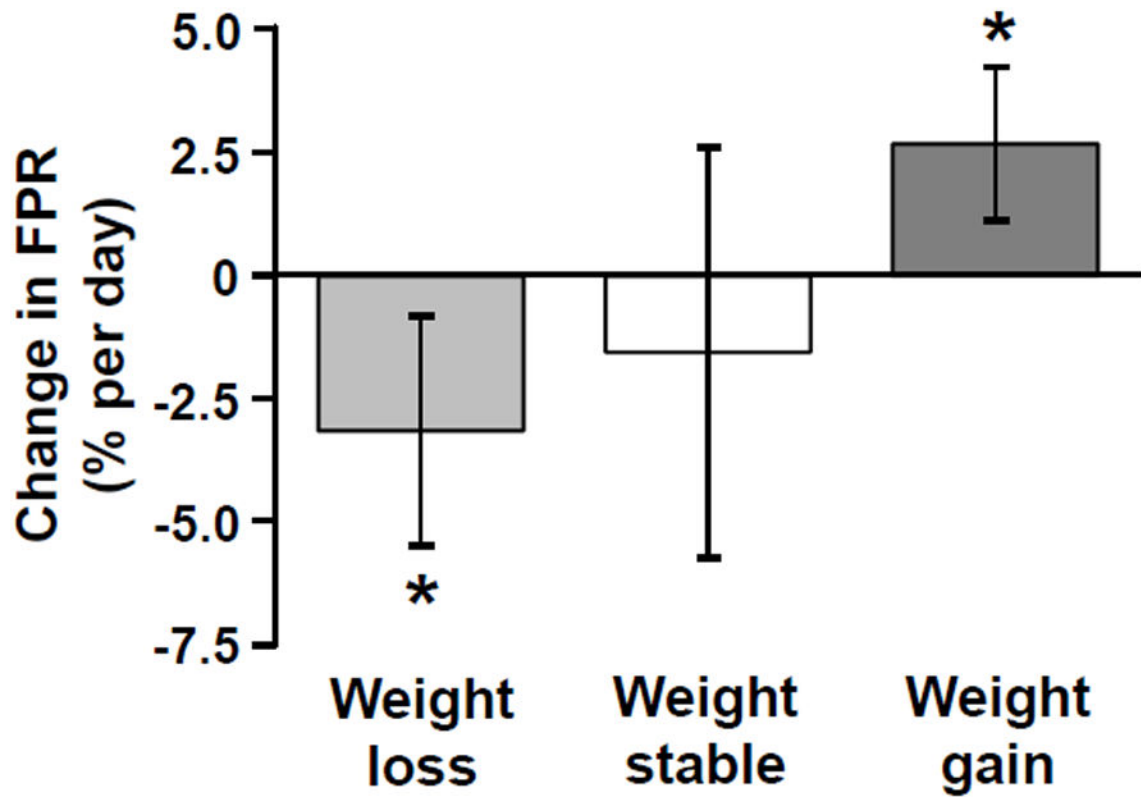
Figure 3.
Changes in *in vivo* colonocyte fractional proliferation rate (FPR) after diet-induced weight loss (n = 15), weight maintenance (n = 7), or diet-induced weight gain (n = 7) in people with obesity. Data are means ± SEM. *The change after weight loss was significantly different from the change after weight gain, after adjusting for baseline FPR and the duration of the diet intervention ($P = 0.045$).
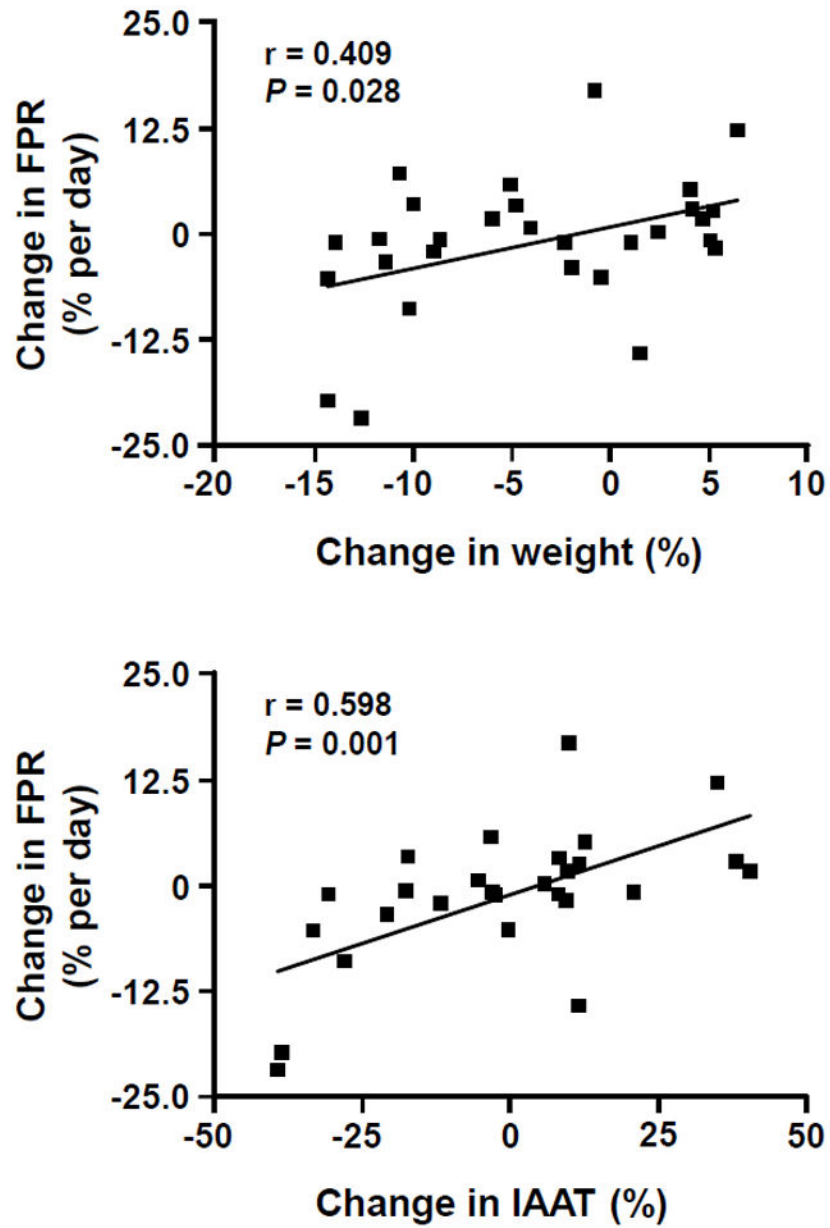
Figure 4.
Relationship between changes in *in vivo* colonocyte fractional proliferation rate (FPR) and diet-induced changes in body weight (*top*) and intra-abdominal adipose tissue (IAAT) volume (*bottom*) (n = 29).