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Resistant Starch from High Amylose Maize (HAM-RS2) Reduces Body Fat and Increases Gut Bacteria in Ovariectomized (OVX) Rats

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Abstract

Objective—Obesity after menopause is a health concern for older females. Changes in the microbiota are likely to occur with this condition. Modifying the microbiota with a prebiotic is a plausible strategy for improving the health of menopausal females.

Design and Methods—Resistant starch type 2 from high-amylose maize (HAM-RS2) was used as a prebiotic in rats in a 2 × 2 factorial study with two levels of HAM-RS2 (0 or 29.7% of weight of diet) referred to as energy control (EC) and HAM-RS2 diets, respectively; and two levels of surgery, ovariectomized (OVX) and sham.

Results—In a 6-week, postsurgery recovery period, OVX rats gained more body weight with consumption of a similar amount of food. Subsequently, consumption of HAM-RS2 versus EC diets resulted in reduced abdominal fat in both OVX and sham rats; but when normalized for disemboweled body weight (body weight minus GI tract), there was no effect of surgery, only reduction with HAM-RS2. Targeted bacterial populations were estimated that are known to ferment HAM-RS2 or metabolize the products of that initial fermentation. OVX and sham rats demonstrated increased bacterial levels with dietary HAM-RS2 for all bacteria. Additionally, culture techniques and qPCR provided similar results.

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Conclusion—This study shows that, as expected, OVX increases adiposity. However, contrary to previous effects seen in obese mice, this did not prevent fermentation of HAM-RS2 and consequently, the fat gain associated with OVX was attenuated.

Introduction

Reduced estrogen production during menopause is believed to be a major cause for increased body fat and one factor contributing to increased risk for obesity in older females (1). Modification of GI microbiota may alleviate obesity by controlling energy expenditure and storage. Widespread use of antibiotics has been studied for modification of GI microbiota (2). This is not an optimal solution for obesity because these are drugs with possible bacterial resistance. Adding prebiotics to the diet as a means for altering gut microbial ecology may be a more viable alternative because they are foods (3).

This study was conducted to examine the effects of type 2 resistant starch from high-amylose maize (HAM-RS2) as a prebiotic on targeted cecal microbial populations in an endocrine model of human obesity, using ovariectomized (OVX) or sham-operated (sham) rats.

Methods and Procedures

Rats and diets

Forty virgin female Sprague Dawley rats were purchased from Harlan (Indianapolis, IN). Twenty rats were OVX and twenty had sham surgery performed by veterinarians before shipment. Rats arrived at 10 weeks of age and were fed the energy control diet (EC) for 6 weeks allowing for recovery from surgery and stabilization of the gut microflora. They were then fed treatment diets for 12 weeks with 10 rats in each of four groups (Table 1). Weights of rats and food intake were measured three times per week. The study was approved by the Louisiana State University IACUC.

Treatment diets used the AIN-93G diet as a base and were formulated to contain 3.3 kcal g^{-1} of metabolizable energy (ME). A 100% amylopectin cornstarch (42.5 wt% of diet), Amioca® (Ingredion Incorporated, Bridgewater, NJ), and increased cellulose (15.6%) were used in the EC diet. The cellulose was used to match the ME content of the EC diet to the HAM-RS2 diet. Hi-maize 260® (Ingredion) cornstarch (53.1%) was used as the source of HAM-RS2. This ingredient was estimated by Ingredion to contain 56% HAM-RS2. The HAM-RS2 diet was estimated to contain 29.7% HAM-RS2. Hi-maize 260® has been estimated to contain 2.8 kcal g^{-1} ME (4).

GI tract collections and culture bacterial analyses

At study end, rats were killed and the GI tract with contents was collected from the base of esophagus to the anus and a suture was used to tie off both ends of the cecum for each rat. The GI tract was weighed and suture weights subtracted. This weight was subtracted from body weight to determine disemboweled body weight (DBW). The cecum and large intestine were weighed together and then the cecum was then separated and transferred to a Whirl-pak bag that was placed in a double zip lock bag with an anaerobic GasPak™ EZ Gas

generating Pouch System (Voiglobal Distribution, Lawrence, KS) and immersed in ice. For bacterial analyses rats one through nine in each group were used to form three pools with three rats each, for a total n of 3.

Lactic acid producing (species of *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Leuconostoc and Bifidobacterium*) and total anaerobic (clostridia and other culturable anaerobes) bacterial counts were enumerated using plates. Ceca and contents were ground in a stomacher (Seward Limited, London, UK) and initially diluted 1:4 with peptone buffer solutions (PBS) and serial dilutions were made. Lactic acid bacteria were enumerated by using de Man-Rogosa-Sharpe Agar (MRS agar) (Difco, Laboratories, Detroit, MI). Reinforced Clostridial agar (Oxoid, Basingstoke, UK) plates were used for anaerobic bacterial counts. The MRS agar plates were anaerobically incubated at 30°C for 48 h and reinforced Clostridial agar plates were anaerobically incubated at 37°C for 3–4 days in a chemically generated anaerobic system using anaerobic GasPak™ EZ in an anaerobic box (Mitsubishi Gas Chemical America, New York, NY). Total colony forming units (CFU) were determined and colonies were isolated and verified by gram staining.

DNA extraction—The 1:4 diluted ceca samples were used for DNA extraction. A QIAamp DNA Stool kit (QIAGEN, Valencia, CA) was used with modification in third step of instructions (samples subjected to three cycles of freeze-thaw in liquid nitrogen and 5 min at 95°C in a water bath to disrupt bacterial cell walls). Purified DNA was quantified using a NanoDrop spectrophotometer and DNA sample extracts were diluted to 1 ng μl^{-1}. Purified DNA was stored in a −80°C freezer until use.

Quantitative real-time PCR

Primers for quantitative PCR (qPCR) from previous reports were used for estimation of *Lactobacillus* species, (5), *Bifidobacterium* species (6), Clostridium cluster IV (7), Clostridium cluster XIVa±b (8), and the total bacterial domain (9). These primers target 16S rRNA genes. Targets of primers were checked using “blast” and intended groups were predominantly represented (>80–90%). The SYBR® Green qPCR assay was used to quantify bacteria using an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Cycling conditions were 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, followed by primer-specific annealing temperature (60°C for all primer sets except 50°C for Clostridium cluster XIVa±b) for 1 min, then 78° C for 30 s. Following amplification, a dissociation step was included to analyze the melting curve of amplified product to determine specificity of the amplification.

Standard curves were created by using serial dilutions of quantified (CFU) pure cultures of *Lactobacillus acidophilus* ATCC 4646 (standard for *Lactobacillus* species), *Clostridium leptum* ATCC 29065 (standard for Clostridial cluster IV), *Clostridium coccoides* ATCC 29236 (standard for Clostridial cluster XIVa±b), *Bifidobacterium longum* ATCC 15708 (standard for Bifidobacterium species) and *Escherichia coli* ATCC 25947 (standard for bacterial domain). Serial dilutions were made and used for overnight cultures (*Lactobacillus acidophilus*, MRS agar, anaerobic; *Clostridium leptum* and Clostridium *coccoides*, clostridial agar, anaerobic; *Bifidobacterium longum*, bifidobacterium agar, anaerobic;

Escherichia coli, tryptic soy agar, aerobic) and extraction of DNA. Then for each dilution, DNA was isolated by the QIAamp method as described above. Cycles to threshold (Ct) values were measured by qRT-PCR, and matched to log CFU. Sample Ct values were compared to the Ct of the standard curves.

Statistical analysis

Data were analyzed as a 2 × 2 factorial with RS (±) and surgery (OVX or sham) as the factors. All nonbacterial dependent variables not normally distributed with significance at $w < 0.05$ using Shapiro-Wilk test were log 10 transformed for statistical analyses. Results were considered statistically significant at $P < 0.05$ and analyses were performed using SAS version 9.3 (SAS Institute, Cary, NC). Data (Table 1) are presented in their raw form as least square means (lsmeans) ± pooled SE.

Results

Food intake during 6 weeks before treatments was not different between sham (1,426.7 ± 22.6 g) and OVX (1,452.1 ± 22.6 g). However, OVX rats (345.5 ± 3.9 g) had a ($P < 0.0001$) greater body weight than sham rats (282.5 ± 3.9 g).

After treatments, OVX rats had greater amounts of abdominal fat and greater DBW; but did not have greater percent for abdominal fat/DBW (Table 1). Rats fed HAM-RS2 had lower abdominal fat, lower percent for abdominal fat/DBW, but the lower DBW only approached ($P < 0.06$) significance. Food intake was greater for rats with HAM-RS2 in the diet, but not affected by surgery.

Because of the type of bacterial collection methods used for culture methods, usual fermentation markers were not measured; therefore, the weight of the cecum plus the remainder of the large intestine with contents was used as a surrogate marker of fermentation. This measurement was greater for rats fed HAM-RS2 and for OVX rats.

Rats fed HAM-RS2 had increased amounts of targeted bacteria measured using either culture techniques or qPCR. The effect of HAM-RS2 also included an increased amount of total bacteria using universal primers for the bacterial domain. OVX rats had lower amounts of targeted bacteria and estimated total bacteria. The exception was the amount of Lactobacillus species measured by qPCR which was similar in both surgery groups with HAM-RS2 feeding. This result was not in disagreement with the culture technique measuring lactic acid bacteria as the latter includes any lactic acid producers including Bifidobacterium species.

Discussion

Results from anthropometric measures and food intake with OVX and sham rats are similar to previously published results with dietary HAM-RS2 (10) and surgical treatments (11). Published reports have demonstrated a different microbiota for obese versus lean rodents and humans as the microbiota may change in the process of becoming obese and the new

microbiota helps maintain the obese state (12). Therefore, OVX rats as a model of endocrine obesity, would likely have a different microbiota than sham rats.

In the current study, targeted bacterial populations were estimated that are known to ferment HAM-RS2 or metabolize the products of that initial fermentation (13,14). Thus, it was expected that HAM-RS2 treatment would be associated with increased levels of the bacterial populations measured. OVX rats demonstrated increased bacterial levels with dietary HAM-RS2; however, their response was reduced compared to sham rats fed HAM-RS2 for all but Lactobacillus species. In a previous study, an obese mouse model failed to ferment HAM-RS2 (15). In the current study, OVX rats fermented HAM-RS2. Further studies are required to determine why mice from the obese mouse model do not ferment HAM-RS2 and OVX rats do. Both culture and qPCR techniques demonstrated a positive response to dietary HAM-RS2 with only partial reduction in OVX versus sham rats. Therefore, beneficial health effects known to be associated with fermentation of HAM-RS2 can be expected in this model of obesity. Although OVX still resulted in increased body fat com- pared to sham, the alteration in targeted microbial populations was associated with a reduction of this increase. If this holds true for postmenopausal women then this population may be healthier with consumption of fermentable fiber.

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TABLE 1

Results for ovariectomized (OVX) and sham surgery rats fed energy control (EC) or resistant starch (RS5HAM-RS2) diets

Group	OVXEC	OVXRS	ShamEC	ShamRS	Pooled SEM	RS	Surgery	RS* surgery
Abdominal fat[a,b](abfat), g	12.3	9.9	10.0	7.6	0.8	0.004	0.01	NS
Disemboweled[b] body weight (DBW), g	331.9	319.6	268.3	260.3	5.2	0.06	0.0001	NS
Abfat/DBW, %	3.7	3.1	3.7	2.9	0.2	0.009	NS	NS
Large. Intestine[b] and cecum with contents, g	7.5	12.2	6.3	9.6	0.7	0.0001	0.002	NS
Food intake[c], g	1,419.0	1,485.1	1,363.0	1,485.3	28.7	0.003	NS	NS
Targeted bacterial results								
Lactic acid producing bacteria[d,e], log CFU	6.4	7.8	7.4	94	0.4	0.001	0.006	NS
Anaerobic bacteria[d,f], log CFU	6.0	7.0	6.9	8.6	0.4	0.005	0.008	NS
Lactobacillus species[g], log CFU	5.7	7.3	5.9	7.3	0.1	0.001	NS	NS
Bifidobacterium species[g], log CFU	6.9	8.6	7.0	9.6	0.3	0.001	0.04	NS
Clostridial cluster IV[g], log CFU	8.4	9.0	8.7	10.0	0.3	0.004	0.02	NS
Clostridial cluster XIVa+b[g], log CFU	6.3	8.4	8.3	9.6	0.4	0.0006	0.008	NS
Bacterial domain[g], log CFU	10.0	12.1	10.3	12.5	0.3	0.005	0.06	NS

[a] Abdominal fat includes four fat groups: mesenteric (attached to GI tract), ovarian (around ovaries and uterus), perirenal (attached to kidney), and retroperitoneal (fat in abdominal cavity not attached to a particular organ).

[b] Data were not normally distributed with significance at $w < 0.05$ with the Shapiro-Wilk test and were log 10 transformed for statistical analyses, and presented in their raw form.

[c] Both EC and RS (HAM-RS2) diets had 3.3 kcal g^{-1} of metabolizable energy.

[d] Measured using bacterial culture techniques.

[e] Includes Bifidobacteria.

[f] Includes Clostridial bacteria.

[g] Measured by qPCR.