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Diet Composition affects surgery-associated weight loss in rats with a compromised alimentary tract

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Abstract

Background—Esophageal adenocarcinoma (EAC) is the fastest growing cancer in terms of incidence and has a high mortality rate. The animal model to study EAC uses esophagoduodenal anastomosis (EDA) to induce mixed-reflux (bile/acid) causing esophagitis, barrett's esophagus and EAC sequence within 6 months. However, the lack of fully functional stomach in these rats leads to the development of malnutrition.

Methods—We have assessed the ability of a chemically pure, purified ingredient diet (AIN-93M) to reduce surgery-associated malnutrition in rats that have undergone the EDA-surgery. Animals were either sham- (SH) or EDA-operated and fed either a grain-based rodent diet (RD) (SH-RD, n=3; EDA-RD, n=10) or a purified diet (PD) (SH-PD, n=4; EDA-PD, n=11). The animals were weighed periodically for assessment of weight gain and euthanized at the end of 24 weeks to measure esophageal tumor incidence.

Results—Animals that underwent sham surgery continued to gain weight throughout the study period and no tumors were detected. The EDA-operated animals had significantly lower weight gain compared with sham animals. There was no significant difference in weight gain among EDA animals fed 2 different types of diets until 9 weeks after the surgery. After 9 weeks, EDA-RD continued to lose weight significantly, whereas the weight loss leveled in EDA-PD ($p<0.001$). At termination, neither tissue histopathology nor tumor incidence was significantly different between the groups.

Conclusion—These results show that compared to a natural ingredient diet, a purified ingredient diet can reduce surgery-associated weight loss in rats with a compromised alimentary tract. This reduction in malnutrition has the potential to reduce the confounding effects of weight loss on future animal studies reported.

Keywords

Esophagoduodenal anastomosis; esophageal adenocarcinoma; grain-based lab rodent diet; purified AIN-93M diet; malnutrition

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INTRODUCTION

According to the most recent analysis of cancer trends by the Center for Disease Control and Prevention, the incidence and mortality rate for many of the common cancers among men have decreased in the last decade (1). However, a notable exception to this trend is esophageal cancer, both the incidence and mortality rates of which have continued to increase steadily over the last 3 decades (1). Esophageal cancer ranks 13th among incident cancer in men but is 7th in cancer cause of death, and is estimated to have been diagnosed in 13,000 men in the year 2008 with an 87% mortality rate (1,2). There are 2 types of esophageal cancer, squamous cell carcinoma (SCC) and esophageal adenocarcinoma (EAC).

The incidence of SCC has been decreasing whereas that of EAC has continued to increase (3,4). Among the risk factors for the development of EAC are age, body mass index (BMI) and smoking (5). These risk factors, independently and synergistically, affect both the development and the effects of the primary risk factor- Gastroesophageal reflux disease (GERD) (6). GERD gives rise to Barrett's esophagus (BE) and EAC incidence is highly associated with the presence of BE (7). Diet is another important risk factor in the development of EAC. Epidemiological studies show that a diet rich in fruits and vegetables decreases, and one rich in saturated fats and meat increases the incidence of esophageal cancer (8,9).

Animal models of disease development are useful in studying the causes, course and control of the disease. Several animal models involving surgical manipulation of the gastroesophageal junction have been proposed to study the effect of acid, bile and mixed (acid and bile) reflux on the development of esophageal cancer (10). These models differ in several aspects of the experimental procedures such as (1) the strain of rats used (Wistar Vs SD); (2) the site of reflux induction (gastroduodenal; duodenal; gastroduodenojejunal; duodenojejunal); (3) presence of portions or all of the stomach (total gastrectomy; antrectomy and pylorotomy) and (4) administration of carcinogens (injected and oral) Reviewed in (10–12). Consequently, a wide variability in experimental outcomes such as the latency period to tumor incidence (20 to 50 weeks) and the type of esophageal carcinomas (pure squamous, mixed adenosquamous and pure adenomatous) have also been reported (10–12). However, 2 things consistent across most studies is the use of male rats and of lab chow, the standard grain-based natural-ingredient diet. Malnutrition resulting from surgical manipulation of the alimentary tract is inherent to these animal models and thus a potentially confounding effect of these results. Although commonly reported, it has not been the focus of most studies. It is important to understand the role of malnutrition especially that of micronutrients in these animals as it may affect the inflammation, initiation and the tumorigenic process, which is the main experimental outcome of these studies. In our laboratory, we have successfully used the animal model for the induction of mixed-reflux in rats using the esophagoduodenal anastomosis (EDA) to study the development of reflux-induced EAC (10); describe the molecular changes that occur during the progression of BE to EAC (13–15) and for chemoprevention studies (16,17).

In studies that investigate the role of dietary components in the prevention of cancer, purified component diets such as AIN-76A and AIN-93M are commonly used due to better quality control (18,19). However, a typical purified diet (PD) differs from a standard rodent diet (RD) in several ways such as source of ingredients, composition, nutritive value and bioavailability of nutrients (20). In addition, soy protein commonly included in RD can affect the digestion of feed by changing pancreatic enzyme secretions (21,22); altering gastric emptying (23) and affect protein digestibility (24) compared to casein or milk protein, which is the source of protein in PD. Thus in the presence of a compromised

alimentary tract as in rats that have undergone EDA surgery, the composition of the diet may play a major role in its digestibility and hence delivery of appropriate nutrition.

We hypothesized that a chemically pure purified ingredient diet will be more bioavailable and hence lead to lower malnutrition and weight loss than the standard rodent diet, without significantly affecting the tumorigenic outcome. To test this, we compared the effect of diet composition on surgery-associated weight loss and incidence esophageal tumors, induced by EDA surgery in male SD rats fed either a standard laboratory diet (RD; Labdiet® 5001) or a purified diet (PD; AIN-93M) for 6 months.

MATERIALS AND METHODS

EDA Surgery and Animal Treatment

Male Sprague-Dawley rats (8 weeks old) were purchased from Harlan, Inc. (Indianapolis, IN) and acclimated for a week before surgery. Food and water were provided *ad libitum*. The EDA-operation was performed on those that weighed over 300 g as described (14). Solid food was withdrawn 24h before surgery. The animals were anesthetized with 60 mg/kg sodium pentobarbital and pedal reflex checked to ensure anesthesia. The gastroesophageal junction was ligated flush with stomach, and the distal esophagus transected proximal to the ligature. An enterotomy (4 mm) was made 1 cm distal to the pylorus on the antimesenteric border. The distal esophagus was then anastomosed to the duodenal enterotomy with accurate mucosal to mucosal opposition. An empty laprotomy without the anastomosis was performed on sham groups. Proper postoperative care was provided to all animals. The animals were euthanized 24 weeks after surgery using carbon dioxide asphyxiation. The esophagus or the anastomosis was removed and checked visually for the presence of tumors. This protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Louisville (KY).

Thirteen animals were fed a grain-based standard laboratory rodent diet (LabDiet® 5001) purchased from PMI international Inc. (Brentwood, MO). Fifteen animals were fed the AIN-93M purified diet purchased from Harlan-Teklad (Madison, WI) and formulated as described (20). All feeds started on arrival to the animal facility prior to surgical manipulation. They were randomized to receive either EDA or sham-surgery as in Table 1. The animals were weighed periodically until 24 weeks. Information on the composition of both the standard rodent diet (RD) and purified diet (PD) can be found on the manufacturer's website at the url-<http://www.labdiet.com/pdf/5001.pdf> and http://www.harlan.com/research_models_and_services/laboratory_animal_diets/teklad_custom_research_diets/ain_formulas.hl.

Tissue Processing for Hemotoxylin Eosin (HE) Staining

The EDA junction was excised and fixed in 10% buffered formalin for 24 h and transferred to 80% ethanol and embedded in paraffin. The paraffin embedded tissues were cut in to serial sections of 5 µm thickness and processed for H and E staining. The HE stained sections were examined for indicators of refluxesophagitis such as inflammatory cell infiltration, basal cell hyperproliferation, papillae hypertrophy, dilation of venules, in-growth of capillaries, epithelial sloughing and ulceration; of BE such as columnar-lined esophagus containing absorptive and goblet cells, with or without dysplasia and of EAC such as dysplasia and invasion of lamina propria (Mueller, et al., 2004; Li et al., 2007).

Statistical Analysis

The weight gain data is presented as mean ± SEM and was analyzed using a two-way Analysis of Variance (ANOVA) followed by a Bonferonni test. A p-value <0.001 was

considered significant. All statistical analyses were performed using Graphpad Prism® software (Graphpad Inc., CA)

RESULTS

Surgery-associated Weight Loss in the EDA-model

The EDA animal model stimulates the presence of a gastro-esophageal mixed reflux. Upon comparing weight gain among animals, it was found that there were no significant differences among sham-operated animals, regardless of the diet (Figure 1). These animals also continued to gain weight throughout the study period. After the initial surgery, reflux-induced animals continued to gain weight at a slower pace, and there was a significant difference ($p<0.001$) between the body weights of sham-rats and EDA-rats, irrespective of the diet, starting at 2 weeks after surgery (Figure 1). Also, there is a significant difference in the amount of feed consumed between the SH- and EDA-operated rats starting 15 weeks after surgery (20 g/rat/day for SH-PD versus 14 g/rat/day for EDA-PD, $p<0.01$; Aiyer, Li and Martin, manuscript in preparation). It must be noted that the feed intake before 15 weeks is not different (unpublished data), yet there is a significant difference in weight gain between SH- and EDA-operated rats. Also, the reduced food intake after 15 weeks could be linked to disease progression or occlusion of the esophagus due to metaplastic changes associated within the EDA model when compared to the SH animals.

Comparison of Weight Loss Between RD and PD

Regardless of the type of diet, the body weight of EDA-operated animals was significantly lower than their sham-operated counterparts at each time point starting at 2 weeks after surgery (Figure 1, $p<0.001$), suggesting that EDA-surgery by itself slows weight gain in these animals. However, there was a significant weight-loss in EDA-RD starting at 9 weeks, whereas there was no weight-loss EDA-PD (Figure 1, $p<0.001$). In fact, the weight loss in this group leveled off at 12 weeks and stayed the same until the end of the study. These results suggest that the diet composition has a significant effect on the surgery-associated weight-loss.

Comparison of Histopathological Changes and Esophageal Tumor Incidence in Animals Fed Either RD or PD

The severity of esophagitis and progression to BE was visually scored for tissue sections collected from each animal. All animals with EDA surgery showed initial basal cell hyperplasia, erosion with inflammatory cell infiltration and papillary hypertrophy. There was no significant difference in the extent of esophagitis or BE in animals fed various diets (Table 1).

SH-operated rats did not develop esophagitis or esophageal tumors, proving that tumors seen in EDA-operated rats are a result of gastro-esophageal reflux. The gross appearance of the EDA operated rats distal esophagus was dilated mucosal layer was thickened, friable and its surface irregular compared to sham-operated controls. Tumors could be grossly visualized in most animals. Further histological examination revealed the presence of extensive esophagitis with squamous hyperplasia, high-grade dysplasia and inflammatory infiltration along with BE changes including replacement of squamous cells with columnar cells and presence of goblet cells (Figure 2B–D). Although it appeared that the esophageal tumor incidence was slightly lower in EDA-PD animals (3 of 11; 27%) compared to EDA-RD animals (4 of 10; 40%), this difference was not statistically significant. The tumors visualized in the stained sections were adenocarcinomas with both atypia and invasiveness (Figure 2D).

DISCUSSION

In this study, we show that the composition of the diet significantly affects weight loss in EDA-operated animals. It can be inferred that this weight loss is surgery-associated as sham-operated animals, regardless of the diet, do not show any difference (Figure 1). Further, we show a difference in tumor incidence in EDA-RD (4/10–40%) compared to the EDA-PD (3/11–27%). Also, we demonstrate a difference between the pathology of the tumors as well as the progression of BE to EAC (Table 1).

In the EDA-operated animals, even though the stomach secretes acid, the storage and digestion function of the stomach is bypassed. Instead, the anastomotic junction usually expands to accommodate the storage and digestion of feed. The primary function of stomach in monogastric animals (including rats, rabbits, pigs, horses and humans) is the initial mechanical and chemical breakdown of food beginning the digestive process. It is established from previous animal models as well as human bariatric surgeries that the presence of the stomach is not absolutely necessary for digestion (10,12). However, malnutrition of certain nutrients is common in the absence of a fully functional stomach.

Standard diets for laboratory rodents are made from a variety of grain and sometimes animal-derived ingredients. They may have varying levels of non-nutrients such as phytochemicals that cannot be controlled. The ingredients found in the RD and PD used in this study is listed in Table 2. The macronutrient composition of the diets is compared. In case of RD, each major ingredient contributes varying amounts of macronutrients (protein, fat, carbohydrate) and a variety of micronutrients. There are several differences in the composition of these diets which applies to both the source and content of the 3 main macronutrients. First, RD has higher protein content (when expressed by weight and as % of kcal) than PD. The percentage of protein calories in RD and PD are 28% and 13.8%, respectively (Table 2). The protein in RD is derived from plant (soybean meal, alfalfa meal, wheat ingredients) and animal sources (fish meal, meat and bone meal, whey). In PD, the protein source is primarily casein, a concentrated protein source. Second, CHO calories in RD are much lower (60%) than that for PD (75%). Similar to protein, CHO source is a mixture of natural ingredients for RD, whereas, PD contains 3 main ingredients –cornstarch, sucrose and maltodextrin. Third, the percentage of fat calories is slightly higher in RD (13.5%) compared to PD (10%). The source of fat in RD is a mixture of saturated (lard as added fat, which is less than half of the total fat) and unsaturated fats (from various natural ingredients used); whereas that in PD is soybean oil, a source of unsaturated fatty acids.. Fourth, RD has a much higher indigestible fiber component than PD. Fiber in RD consists of cellulose, hemi-cellulose and lignin, and comes from the ingredients themselves – not added as cellulose as in PD. Further, the 5% value listed for RD is for crude fiber only. A better benchmark of total fiber is the value for NDF (neutral detergent fiber) given in the datasheet as 15%. Further, many ingredients found in RD are also sources of phytoestrogens (Table 2). Soybean meal is a well documented source of daidzein and genistein and alfalfa sprouts contain coumesterol (25,26). Lignin, a component of the fiber in RD is a precursor of the phytoestrogen lignan in rat gut (27).

The interplay between the absence of a fully functional stomach and the diet composition, ultimately resulting in reduced availability of nutrients, may explain our results. Rats undergoing EDA surgery have been reported to have lower serum iron, albumin and fat-soluble vitamin levels (11,28). The rats were fed lab chow in these studies (28,29). However, the same investigators report a lack of malnutrition in a study using AIN-93M diet in the esophagogastrroduodenal anastomosis (EGDA) animal model in which the stomach is fully functional (30). Thus it is not clear whether reduced malnutrition in these animals was due to use of a purified diet or the presence of a fully functional stomach. Further, these

investigators used intraperitoneal iron injections to promote EAC, adding another layer of complexity to their model.

In our study, we compared the effect of two different diets in the EDA model without iron supplementation. Thus, the confounding effect of both iron supplementation and the presence of a functional stomach are absent. Hence, it is easy to illustrate the effect of diet on the development of inflammation, initiation, and reflux-induced EAC in the EDA model. The progressive weight loss in RD-fed animals suggests the presence of malnutrition. The absence of such weight loss in PD animals can be attributed to the chemically pure nature of this diet, which can be easily digested and assimilated. It has been shown that protein from natural sources such as soy is digested less efficiently than milk protein casein, which is the primary protein source in PD (Table 2) (31,32). Also, non-nutrient substances in RD can inhibit the absorption of micronutrients such as iron (33). Another aspect of RD is the presence of phytoestrogens (Table 2). Reports show that natural ingredient diets may contain more than 800-times the amount of isoflavones found in purified diet (34). However, it cannot be claimed that phytoestrogens play a role in the EAC tumorigenesis without further studies. However, this data presented does demonstrate the variability that the type of diet administered can have on the clinical well-being of the animal and thus the potential effects this may play in future animal models results.

There is an ongoing debate regarding which animal model is best suited to mimic the human condition, with unique pros and cons associated with each. The advantages of the EDA model from our studies are 1) relatively short period of tumor induction (40% tumors in 24 weeks versus 40 weeks reported by others) (14,17); 2) ability to induce mixed –acid and bile- reflux; 3) similarity of pathophysiology to human BE and EAC. The limitations are 1) presence of malnutrition and weight loss due to the lack of stomach and 2) confounding effects of this malnutrition, if present, on tumor incidence. However, this initial report suggests that this malnutrition can be eliminated simply by the use of a purified diet and almost similar tumor incidence can be achieved. Further, the use of a PD also enables us to conduct dietary chemoprevention studies.

In summary, these results suggest that feeding a purified diet may significantly prevent surgery-associated weight loss in EDA operated rats compared to feeding rodent chow. To dissect the effects of disease progression versus malnutrition alone, the effects of these diets in an esophagoduodenogastric anastomosis (EGDA) model, in which a fully functional stomach is present, have to be studied. The results will demonstrate whether the weight loss seen in the EDA model is associated with malnutrition due to poor digestibility of the RD itself due to absence of a stomach or whether there are other components in RD that can accelerate the disease process. Currently we are performing a larger study to confirm these initial results and determine whether diet composition significantly affects tumor incidence. We are also conducting systematic blood analysis to confirm that there is micronutrient and protein deficiency in RD fed animals.

In conclusion, this study provides initial evidence that use of a purified diet can prevent surgery-associated weight loss in the EDA model and may warrant the use of such diets in animal models with manipulation of alimentary tract where digestion and absorption of feed may be compromised.

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Figure 1. Effect of diet and EDA-surgery on body weight gain in male SD rats

Rats were fed either RD (solid symbols) or PD (open symbols) and operated with SH- (circles) or EDA- surgery (squares). The surgery was performed on week 0 and weight gain was recorded periodically for 24 weeks. SH-RD (solid circles); EDA-RD (solid squares); SH-PD (open circles) and EDA-PD (open squares). #- Significantly different from SH-operated animals on respective diets ($p < 0.001$) § - Significantly different from EDA-PD ($p < 0.001$)

Figure 2. A–D Histological changes in the esophageal epithelium after esophagoduodenal anastomosis (EDA) in rats fed purified diet

A- Normal esophagus in SH-PD rats; B- esophagitis with hyperplastic epithelium, papillary hypertrophy and inflammatory infiltration; C- Specialized Intestinal Metaplasia (Barrett's Esophagus) with columnar and goblet cells with islands of squamous cells and D- Esophageal Adenocarcinoma with atypia and invasion of lamina propria in EDA-PD rats (Hematoxylin and eosin staining, 200X magnification). Sequential histological changes occurring in EDA-RD group have been published (14).

Table 1

Comparison of histological changes and tumor incidence

Study	Diet	Surgery	n	Esophagitis	Animals (%incidence)		
					BE	EAC	
1 ^a	RD	Sham	3	0	0	0	
		EDA	10	3.0 ± 0.9	9 (90%)	4 (40%)	
2	PD	Sham	4	0	0	0	
		EDA	11	2.8 ± 0.4	9 (81%)	3 (27%)	

Data represents mean ± SD for all animals. Esophagitis was scored based on the Hetzel-Dent system (Grade 0- Normal appearing mucosa; grade 1- basal cell hyperplasia, mucosal edema, hyperemia, mucosal friability; grade 2- superficial erosion involving <10% of mucosal surface of the last 5 cm of the esophageal squamous mucosa; grade 3- superficial erosions or ulcerations involving 10–50% of the mucosal surface and grade 4- deep peptic ulceration and confluent erosion of >50% of the esophageal squamous mucosa) on 5 random regions for each sample and the scores averaged. BE was assessed based on the presence of columnar lined esophagus consisting of absorptive and goblet cells. EAC was identified by dysplastic glandular cell growth with both atypia and invasiveness. **a-** Results from this study are published (14).

Table 2

Comparison of macronutrient composition of standard rodent and purified diets.

Macronutrient	Rodent diet (Labdiet 5001®)		Purified diet (AIN-93M diet)	
	%kcal	Primary source	Contains phytoestrogen	% kcal
Protein		Soybean meal	Yes	
		Dried alfalfa meal	Yes	
	≥ 23%	Wheat germ	-	14%
		Porcine meat meal	No	Casein
		Fish meal	No	No
		Dried whey	No	
Carbohydrate		Ground Corn	No	
		Beet pulp	No	Purified cornstarch
	60%	Ground oats	No	
		Cane molasses	No	75% Maltodextrin
		Wheat middlings	No	
		Wheat germ	-	Sucrose
Fat	13.5%	Porcine animal fat (Lard)	No	10%
				Soybean oil
Fiber*	5%	Cellulose, hemicellulose and lignin	Yes	5%
				Cellulose
				No

Information presented in the table was collected from manufacturer's hand out for LabDiet® 5001 available on the PMI International website (<http://www.labdiet.com/pdf/5001.pdf>) and for AIN-93M diet on the Harlan Teklad website (<http://www.tekladcustomdiets.com/ain.asp>).

* Fiber (crude) contains cellulose, hemicellulose and lignin from natural sources in the rodent diet. Lignin is converted to lignans, which are estrogenic, in the rat gut (27).