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A CLONED PIG MODEL FOR EXAMINING ATHEROSCLEROSIS INDUCED BY HIGH FAT, HIGH CHOLESTEROL DIETS

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The pig is a recognized model for the onset of coronary heart disease and heart attacks. Previous studies have shown that serum cholesterol levels in the pig can be elevated using a high fat, high cholesterol (HFHC) diet. What has been lacking is a genetically defined model corresponding to human ApoE4 susceptibility that can be linked to diets capable of inducing atherosclerosis. This study used a cloned pig model to examine the impact of cholesterol levels with the development of aorta fatty deposits leading to atherosclerosis. Diets were formulated using vegetable sources of protein to provide similar intakes of metabolizable energy, calcium, phosphorous and principal amino acids in both control and HFHC groups. After 60 days, the HFHC group demonstrated a 40-fold increase in aortic fatty streak lesion area combined with 6- and 11-fold increases in total and LDL cholesterol, respectively, over control diet fed cloned pigs. Previous studies have suffered from either imbalanced total caloric intake, an overall imbalance in the nutrition of the control versus HFHC groups or genetic heterogeneity when evaluating dietary constraints related to atherosclerosis. This study demonstrated that cloned, genetically-defined ApoE4 pigs provided balanced nutrition diets provide an experimental system ideally suited to examining atherosclerosis and the onset of coronary heart disease.

Keywords: Atherosclerosis; Cholesterol; Cloned pig; Diet

INTRODUCTION

The pig has witnessed an increased use in recent years for heart studies due to its similar coronary anatomy and physiology to the human heart.[1,2] One example is

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the similarity between the collateral flow of the pig heart to that observed in humans. Pigs blood flow is also more similar to the human heart than other experimental systems such as the mouse or dog.[3–5] The utility of the pig model has already proven an advantage for examining myocardial infarction (MI) repair therapies.[6–11] While very informative, successful therapies in animal models often do not translate to successes in human clinical trials. In part, this is due to the use of healthy animals for creating damage models. Such systems often do not reflect secondary effects of poor diets, smoking, and high blood pressure present in human subjects in clinical settings. For example, while coronary atherosclerosis has been the major cause of MI, its possible impacts on repair therapies are not represented in animal models.

The pig provides an excellent model for atherosclerosis. Pigs develop spontaneous atherosclerosis with increased age and have lipoprotein profiles and metabolism similar to humans.[12–14] The use of high fat, high cholesterol diets in pigs results in elevation of total and LDL plasma cholesterol.[13] These studies have shown the ability to induce aortic lesions very similar to those seen in human atherosclerosis disease.[14–16] In humans, analysis of the apolipoprotein E (APOE) gene shows single nucleotide polymorphisms (SNPs) resulting in amino acid substitutions at residues 112 and 158 that are responsible for the e2, e3, and e4 isoforms. The translated protein with cysteine to arginine substitutions at both positions 112 and 158 results in the ApoE4 isoform, which is associated with a predisposition for higher plasma LDL-cholesterol.[17] Sequencing of porcine APOE has determined high amino acid identity in the critical APOE receptor binding sites with arginine residues at positions 112 and 158, corresponding to the ApoE4 isoform.[18,19] We have recently analyzed the APOE pig gene for single nucleotide polymorphisms (SNPs) in exon 4 of the gene. We have examined sequences from 128 pigs representing 11 different breeds and have found that all samples were homozygous for the ApoE4 isoform (unpublished results). This finding lends weight to the use of the pig as a model for atherosclerosis induced heart failure due to the use of ApoE4 as a predictor for chronic heart disease in humans.[20–22]

The goal of this work was to design a HFHC diet to determine the impact of elevated plasma cholesterol levels, using a cloned pig model, on the formation of atherosclerotic plaques. Thus, control and atherogenic diets with similar intakes of metabolizable energy, calcium, phosphorous, and principal amino acids were formulated. Due to the high caloric content of the fat additives in the HFHC diets, the ratios of corn and soybean meal, including supplements, were adjusted to balance the nutrients of both diets. The goal of this model is to explore the impact of atherosclerosis on cardiac damage and to support subsequent pharmaceutical and cell-based therapeutic protocols.

MATERIALS AND METHODS

Animals and Diets

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Illinois. ApoE4 homozygous Duroc gilt (2–14) was used as the genomic template for producing cloned animals using somatic cell nuclear transfer. Four Duroc female pig clones from the same litter, 14 months

old, were randomly assigned to one of two treatment groups; two clones were fed a control diet (CD) and two clones were fed a high-fat, high-cholesterol (HFHC) diet. Supplements for the HFHC diet were obtained from Research Diets (New Brunswick, NJ). Pigs were fed once daily in the morning and had free access to water. Pigs were maintained on the diet for 60 days.

Control and HFHC diets (Table 1) were formulated and provided feed allotments so that both treatments provided similar daily intakes of metabolizable energy and nutrients. The atherogenic diet was supplemented with 2.0% cholesterol, 17% hydrogenated coconut oil, 2.3% corn oil, and 0.7% sodium cholate.[13] Total metabolizable energy was controlled by adjusting the daily rations. To balance the nutritional intake of both the control and HFHC regimens, the ratios of corn, soybean meal, and other additives were adjusted to provide similar levels of calcium, phosphorus, and the principal amino acids lysine, methionine, threonine, and tryptophan in both diets (Table 1). The two experimental diets were formulated to provide nutrients in approximately the same ratio to metabolizable energy while meeting required NRC minimal levels.[23]

Plasma Lipids

Blood samples were collected via the jugular vein from pigs fasted overnight, 120 days before initiating the experiment (day 0) and every two weeks until the end of the study and coded for pathology analysis. Coded plasma samples were sent to Mayo Medical Laboratories for lipoprotein analysis (Rochester, MN). The lipoprotein metabolism profile reported total cholesterol, total triglycerides, quantification of cholesterol and triglycerides in VLDL, HDL cholesterol, and LDL cholesterol.

Table 1 Diet compositions

Ingredients	Control diet (CD) weight, g/100 g	High fat/cholesterol diet (HFHC) weight, g/100 g
Corn	85.94	50.89
Soybean Meal	10.81	22.82
Limestone	1.07	1.40
Calcium Phosphate	1.61	2.11
Premix minerals	0.35	0.45
Premix vitamins	0.20	0.26
Tylan-40	0.03	0.03
DL-Methionine	0	0.03
Coconut oil	0	17.00
Corn oil	0	2.30
Cholesterol	0	2.00
Sodium cholate	0	0.70
Metabolizable Energy	3.304 kcal/g	4.239 kcal/g
Daily Feed Intake	2 kg/day	1.5 kg/day
Metabolizable Energy Intake	6,608 kcal/day	6,359 kcal/day

Aortic Lesion Analysis

Immediately after collection, the aortas were dissected intact from the thoracic and abdominal cavities from the base of the heart to the bifurcation of the iliac arteries in the pelvis. They were opened longitudinally to expose the intimal surface and rinsed gently with saline. Quantification of fatty streaks was performed using a Sudan IV stain. Aortas were incubated in a solution of 5 mg/mL Sudan IV in acetone and 70% ethanol for 15 min, rinsed with 80% ethanol for 5 min, followed by a rinse under tap water. The aortas were then photographed for scoring. The photograph for each animal was captured via an image scanner to analyze the surface area of atherosclerotic lesions and of the aorta itself using image analysis software (MCID Image Analysis 7.0). The aortic lesion area was expressed as a percentage of stained area to total area.

Collagen and Fat Histochemistry

Sections of aortic tissue 1–2 cm in length were trimmed from the most intense Sudanophilic portions of the artery, placed in tissue cassettes, and fixed in 10% neutral-buffered formalin for 5 days. Aortic segments were embedded in paraffin, cut, mounted on glass slides, and stained using H&E for inflammation and Masson's trichrome for collagen. Similar sections of aorta were flash frozen and stained with oil-red-O for fat.

Statistical Analyses

Data is presented for individual animals and the mean value for a given condition and the corresponding standard deviation are provided.

RESULTS

Plasma Levels of Cholesterol

While on the test diets, the animals on control diets gained 7 and 11 kilograms (an average increase of 5% from initial weight) while the animals on HFHC diets gained 16 and 23 kilograms (an average increase of 12% from initial weight) (Figure 1). Total plasma cholesterol and triglyceride profiles prior to and during the two different diet regimens were determined (Figure 2 and Table 2). Prediet baseline values were 81 ± 5 mg/dL total cholesterol (mean $\pm$ SD, $n = 4$), 48 ± 6 mg/dL LDL cholesterol, and 30 ± 2 mg/dL HDL cholesterol. In pigs fed the HFHC diet, total cholesterol levels increased to 471 ± 18 mg/dL (6-fold increase over control), LDL cholesterol levels increased to 404 ± 24 mg/dL (11-fold increase over control), and HDL cholesterol increased to 56 ± 5 mg/dL (approximately 1.5-fold increase over control) after 8.5 weeks on the experimental diets.

After 60 days on either the control or HFHC diets, levels of total plasma triglycerides were similar for both the control and HFHC groups ($p > 0.4$). This phenotype of elevated total and LDL cholesterol with similar levels of plasma triglyceride relative to control is characteristic of values seen for humans with Type IIa hyperlipidemia.[24]

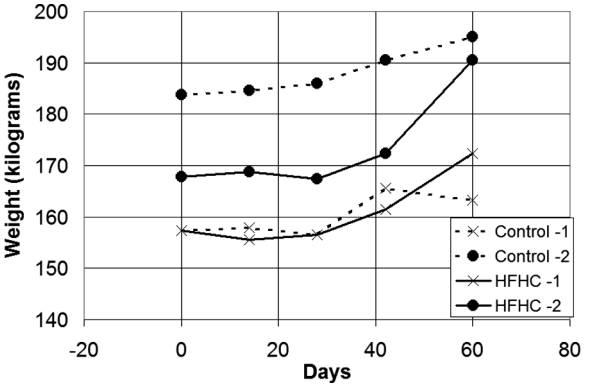

Figure 1 Body weight of adult pigs on control and high fat, high cholesterol diets over the course of 60 days. Body weight over the course of the experiment for pigs on the control or high fat, high cholesterol (HFHC) diets. Pigs were fed a standard diet from birth to 14 months and switched to control or HFHC diet on day 0. All animals received similar caloric intake.

Aortic Lesion Analysis

The atherogenic HFHC diet induced plaque formation in the aortas that was associated with accumulation of LDL cholesterol, whereas feeding of the CD did not produce plaque formation. Figure 3A and E illustrate the accumulation of lipid, stained with Sudan IV, in fatty streaks at 60 days after HFHC diet (A), but not after control diet (E). The stain ratio was calculated as a percentage using MCID Image Analysis 7.0 Software. The aortic fatty streak lesion area (percentage

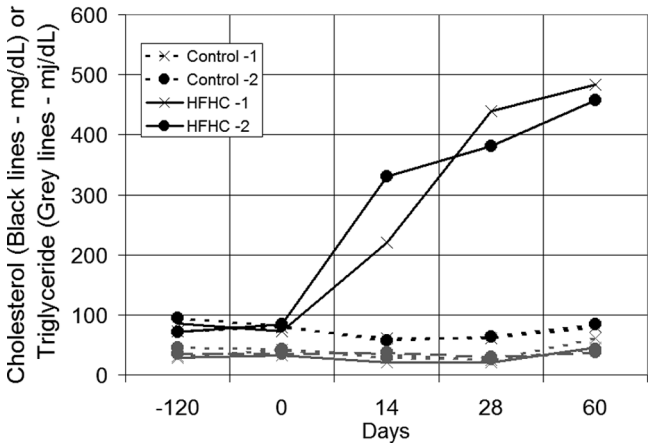

Figure 2 Plasma cholesterol and triglyceride levels of pigs on control and high fat, high cholesterol diets. Total plasma cholesterol (black lines) and triglyceride (grey lines) levels for pigs fed on control and high fat, high cholesterol (HFHC) diets. Pigs were fed a standard diet from birth to 14 months. Baseline levels were measured 120 days prior to changing diets and immediately prior to changing diets (day 0). All animals received similar caloric intake.

Table 2 Plasma cholesterol and triglyceride levels and lipoprotein cholesterol levels in swine fed control diet (CD) and high fat, high cholesterol diet (HF)

	Total cholesterol, mg/dL				Total trigyceride, mj/dL			
	HF-1	HF-2	CD-1	CD-2	HF-1	HF-2	CD-1	CD-2
Day −120	86	71	73	94	28	35	31	46
Day 0	73	84	79	82	33	35	41	43
Day 14	220	330	61	57	21	38	34	28
Day 28	439	381	61	63	21	30	24	26
Day 60	483	458	80	84	46	38	61	43
	VLDL cholesterol, mg/dL				VLDL triglyceride, mj/dL			
	HF-1	HF-2	CD-1	CD-2	HF-1	HF-2	CD-1	CD-2
Day −120	16	18	15	3	16	18	15	25
Day 0	3	1	2	3	27	20	33	34
Day 14	18	25	5	5	12	26	26	21
Day 28	32	29	6	7	9	19	17	19
Day 60	32	19	12	10	20	19	34	32
	LDL cholesterol, mg/dL				LDL triglyceride, mj/dL			
	HF-1	HF-2	CD-1	CD-2	HF-1	HF-2	CD-1	CD-2
Day −120	51	44	45	53	3	1	9	5
Day 0	40	53	43	46	2	4	4	5
Day 14	150	234	31	32	6	8	5	5
Day 28	337	300	30	30	9	9	4	4
Day 60	421	387	35	39	19	15	16	8
		HDL cholesterol, mg/dL						
		HF-1		HF-2		CD-1		CD-2
Day −120		32		25		27		38
Day 0		30		30		34		33
Day 14		52		71		25		20
Day 28		70		52		25		26
Day 60		59		52		33		35

of whole area) was $29 \pm 5\%$ for the HFHC group and $0.7 \pm 0.1\%$ for the CD group (see Figure 4), a 40-fold difference. The increase in plasma LDL alone suggests it may be sufficient to initiate the development of early atherosclerotic fatty streak lesions by inducing endothelial dysfunction and an increased permeability of the vessel wall.[25]

Using sections taken from areas identified by Sudan IV staining, histological staining by the Hemotoxylin & Eosin method (Figure 3B, 3F) showed no inflammatory cell infiltration in the vascular wall after 60 days on either diet. In addition, staining by the Masson's trichrome method (Figure 3D, 3H) revealed no fibrinogen infiltration in the vascular wall. However, Oil Red O stain (Figure 3C, 3G) confirmed lipid infiltration into the endothelial cells of the intimal layer.

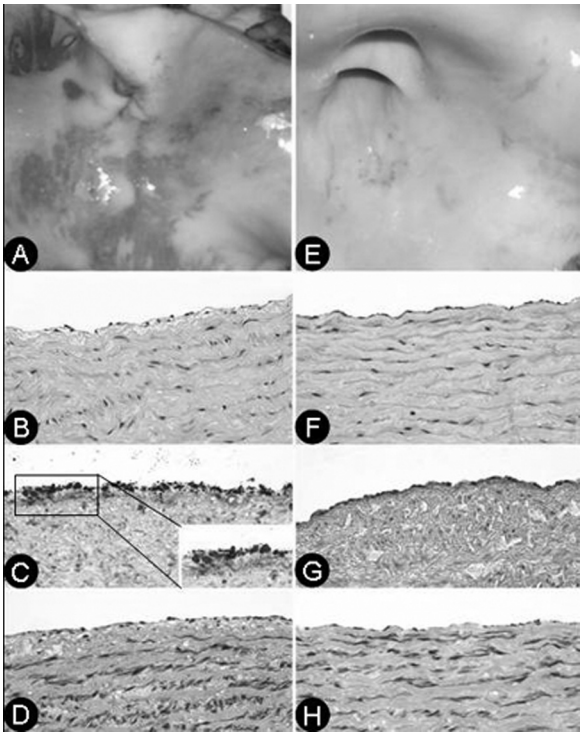

Figure 3 Aortic lesion analysis after 60 days of either control or high fat, high cholesterol diet. Aortic lesion analysis after 60 days on either the HFHC (A-D) or control (E-H) diets. Samples stained with Sudan IV show greater levels of fatty deposits in animals fed with HFHC (A) than control (E) diet. Cross sections of Sudan IV stained regions were further analyzed. Little or no inflammatory cell infiltration after H&E staining is seen in either HFHC (B) or control (F) animals. Lipid infiltration is seen after oil-red-O staining in HFHC (C) but not control (G) animals. After Masson's trichrome staining for collagen, there is little or no fibrosis seen in either HFHC (D) or control (H) animals.

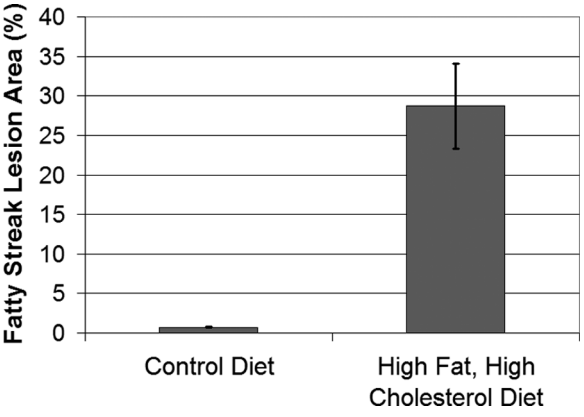

Figure 4 Aortic fatty streak lesion area analysis. The percentage of fatty tissue (Sudan IV stained) is expressed as a percentage of the entire tissue area. Data are shown for pigs on the control and high fat, high cholesterol diets for 60 days. Error bars represent standard deviation of the mean value.

DISCUSSION

This study demonstrated that atherogenic diet fed to genetically pre-disposed adult Duroc gilts results in an increase in plasma levels of total and LDL cholesterol within 60 days, which is associated with the generation of fatty streaks in the aorta. The 6-fold increase in total plasma cholesterol concentration and 11-fold increase in plasma LDL cholesterol concentration lead to high intimal LDL concentrations and subsequent entry of lipid into the artery's subendothelial compartment. These findings suggest that this protocol provides a model in which the earliest stage in atherosclerotic cardiovascular disease can be observed within 60 days on the HFHC diet. Based on the histological analysis of the aorta and the absence of inflammation or fibrosis via Sudan IV staining, the lesions had not yet reached a chronic stage. Thus, this model provides an important tool to evaluate diet-induced vascular disease and to develop intervention strategies. The use of cloned animals whose genome has been sequenced facilitates a more controlled and repeatable experimental system that requires fewer animals than necessary using outbred lines. Variations seen with different treatments may be ascribed to intrinsic biological variations and not genetic differences between animals. While it may be argued that the use of a clone model does not translate directly to a genetically diverse clinical population, this system provides an important tool to address questions pertaining to the relative impacts of different treatment modalities more quickly while using fewer animals than a standard model would allow.

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