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Effect of Different Obesogenic Diets on Pancreatic Histology in Ossabaw Miniature Swine

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Abstract

Objective—Obesity is a factor in the outcome and severity of pancreatic conditions. We examined the effect of hypercaloric diets on the pancreata of Ossabaw swine, a large animal model of metabolic syndrome and obesity.

Methods—Swine were fed with 1 of 4 diets: high-fructose (n = 9), atherogenic (n = 10), modified atherogenic (n = 6), or eucaloric standard diet (n = 12) for 24 weeks. Serum chemistries were measured, and pancreata were examined for histological abnormalities including steatosis, inflammation or fibrosis, insulin content, and oxidative stress.

Results—The fructose, atherogenic, and modified atherogenic diet groups exhibited obesity, metabolic syndrome, islet enlargement, and significantly increased pancreatic steatosis (22.9% ± 7.5%, 19.7% ± 7.7%, and 38.7% ± 15.3% fat in total tissue area, respectively) compared with controls (9.3% ± 1.9%; *P* < 0.05). The modified atherogenic diet group showed significantly increased oxidative stress levels as evidenced by elevated serum malondialdehyde (3.0 ± 3.3 vs 1.5 ± 0.3 μmol/L in controls; *P* = 0.006) and pancreatic malondialdehyde (0.1 ± 0.12 vs 0.04 ± 0.01 nmol/mg protein in controls; *P* = 0.01). None of the swine exhibited pancreatitis or cellular injury.

Conclusions—Ossabaw swine fed with a modified atherogenic diet developed significant pancreatic steatosis and increased oxidative stress, but no other histological abnormalities were observed.

Keywords

animal model; fatty pancreas; metabolic syndrome; fatty liver

Obesity paired with hypertension, insulin resistance, and dyslipidemia—the metabolic syndrome—is a growing epidemic and is linked closely with other chronic diseases. In humans, obesity is a risk factor for fat accumulation in the pancreas and is a predictor of the severity of acute pancreatitis,^{1,2} and small animal models have confirmed these findings. For example, leptin-deficient obese mice were found to have significantly more total pancreatic fat, a condition termed nonalcoholic fatty pancreas disease (NAFPD).³ In another study, the severity of chemical-induced acute pancreatitis was greater in obese mice as compared to lean control mice.⁴

Ossabaw miniature swine are an excellent large animal model for investigating metabolic syndrome and associated conditions.⁵ These swine exhibit a thrifty genotype that allows for the storage of large amounts of fat for survival during famine.⁶ When fed with high-calorie and high-fat atherogenic diets, swine develop several characteristics of metabolic syndrome, including dyslipidemia, obesity, hypertension, and insulin resistance.⁷ Recently, we reported that feeding swine with a modified atherogenic/nonalcoholic steatohepatitis (NASH) diet consisting of cholesterol and fat calories from hydrogenated soybean oil, coconut oil, and lard induced abnormalities in liver histology that closely resemble those observed in human NASH.⁸

Because of the tendency of Ossabaw swine to develop obesity and metabolic syndrome, in this study, we evaluated whether swine also exhibited NAFPD and/or nonalcoholic steatopancreatitis (NASP) when fed with high-fructose, atherogenic, or modified atherogenic (NASH) diets.

MATERIALS AND METHODS

Thirty-seven miniature swine aged 5 to 10 months at the start of the study were allocated to 1 of 4 different diet groups for 24 weeks. The control group consumed a eucaloric diet (2500 kcal/day) and maintained normal body weight, whereas the other 3 groups consumed a hypercaloric diet (6000 kcal/day) that induced obesity. These 37 swine were included in an earlier publication that described a large animal model of diet-induced steatohepatitis and metabolic syndrome.⁸ The 4 groups of diets were the following:

Standard Chow (Control Group, n = 12): These swine received standard chow consisting of 18.5% calories from protein, 71% calories from carbohydrates, 10.5% of calories from fat, and normal concentrations of methionine and choline (3500 and 1500 ppm, respectively).

Fructose Diet Group (n = 9): This high-fructose, normal-fat diet consisted of 20% calories from fructose and 10.5% calories from fat, with methionine and choline concentrations at 2800 and 1200 ppm, respectively.

Atherogenic Diet Group (n = 10): The high fructose-containing atherogenic diet consisted of 20% calories from fructose, 43% calories from fat derived from hydrogenated soybean oil, and methionine and choline at concentrations of 2100 and 900 ppm, respectively.

Modified Atherogenic Diet group (NASH diet, n = 6): The fructose-based atherogenic diet (5B4L; custom formulated by Purina TestDiet, Inc, Richmond, Ind) provided 18% calories from fructose, 17% calories from protein (added casein), 43% calories from fat (admixture of hydrogenated soybean oil, coconut oil, and lard), and methionine and choline at concentrations of 3500 and 700 ppm, respectively. For the remainder of the manuscript, this diet will be referred to as NASH diet.

Animals were given free access to feed for 6 hours a day and unlimited access to water. Animals were humanely killed by excision of the heart under general anesthesia as described elsewhere.⁹ All protocols involving animals were approved by an Institutional Animal Care and Use committee and complied with the recommendations outlined by the National Research Council and the American Veterinary Medical Association Panel on Euthanasia.^{10,11}

Phenotypic and Laboratory Measurements

Body weights were obtained at the beginning of the study and at weekly intervals thereafter. An intravenous glucose tolerance test was conducted on the swine 1 week before the animals were killed, and insulin resistance was assessed by the homeostatic model assessment method.⁸ Total cholesterol, triglycerides, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol were measured in plasma samples using standard methods.⁷ Serum chemistries were measured by a local clinical laboratory (Antech Diagnostics, Fishers, Ind). Fasting serum levels of leptin were measured by a commercial laboratory (Millipore Corp, St Charles, Mo), and serum adiponectin was measured by mass spectrometry and expressed as relative protein intensity (Monarch LifeSciences, Indianapolis, Ind).

Tissue Preparation and Histological Analyses

At the time the swine were killed, a portion of the head of the pancreas was fixed in formalin, processed, and embedded in paraffin for subsequent hematoxylin and eosin (H&E) staining. Immunohistochemistry was also performed on sections cut from paraffin-embedded tissue. Briefly, the sections were deparaffinized, endogenous peroxidase activity was quenched using hydrogen peroxide, and heat-induced antigen retrieval was performed. Sections were incubated with anti-insulin antibody (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif). After incubation with a peroxidase conjugated secondary antibody (Vector Laboratory, Inc, Burlingame, Calif), the reaction was developed using the Vector NovaRed peroxidase substrate kit (Vector Laboratories, Inc, Burlingame, Calif).

Both stains were blindly examined via light microscopy and scored by an expert pathologist (R.S.). The presence of cellular injury, such as inflammation or fibrosis, was evaluated. In addition, islet size and the percentage and placement of β cells within islets were recorded. Islet size was scored based on the insulin-positive area of islets on a scale ranging from zero (normal, no enlargement) to +++ (very enlarged).

The amount of fat in the pancreatic tissue was examined using H&E-stained slides and digitally quantified using SPSS Sigma Scan Pro 5.0 software (SPSS Inc, Chicago, Ill) as described previously.¹² Pancreatic fat was expressed as a percent of total tissue area.

Oxidative Stress Analyses

Two measures of oxidative stress, malondialdehyde (MDA) and trolox equivalent antioxidant capacity (TEAC) were quantitated in pancreatic tissue and serum that had been flash frozen in liquid nitrogen at the time of sacrifice and stored at -80°C until analysis. Malondialdehyde levels in serum and pancreatic tissue homogenates were measured using high-performance level chromatography with UV detection as described previously with some modifications.^{13,14} The total antioxidant capacity in pancreatic tissue homogenates was measured using the TEAC assay described previously with some modifications.¹⁵

Statistical Analysis

Statistical Package for the Social Sciences (SPSS) Version 16.0 for Windows (SPSS, Chicago, Ill) was used to perform statistical analyses. Student *t* tests were used to detect differences between groups, and $P < 0.05$ was considered significant.

RESULTS

Pig Phenotypes

Selected characteristics of the 37 swine in the different diet groups examined in this study are shown in Table 1. Compared to the lean control group, swine in all 3 hypercaloric diet groups had gained a significant amount of weight by the time the animals were killed. Swine fed with atherogenic or NASH diet had significantly elevated serum levels of total cholesterol and low-density lipoprotein cholesterol compared with the control and fructose diet groups, and the NASH diet group had a significantly increased triglycerides compared with all 3 other diets. The NASH diet group also had significantly greater aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase levels compared with the control group as described by Lee et al.⁸ There were no significant differences in amylase, lipase, glucose, or insulin values among any of the diet groups. The NASH diet groups had a significantly increased homeostatic model assessment method value when compared with the control (4.1 ± 1.0 vs 2.1 ± 0.4 , $P < 0.05$). There was a trend for reduction of serum adiponectin in the NASH diet group compared to the controls, but this did not reach statistical significance [$13,296 \pm 663$ vs $16,351 \pm 1322$ quac (expressed as protein intensity), $P = 0.08$], and serum leptin levels in the NASH diet group were significantly greater than in the control, fructose, and atherogenic diet groups (Table 1).

Histological and Morphological Analyses

As shown in Table 2, the amount of fat in pancreatic tissue (measured as a percentage of total tissue area) in the NASH diet group ($38.7\% \pm 15.3\%$) was significantly greater than in the control, fructose and atherogenic diet groups ($9.29\% \pm 1.94\%$, $22.9\% \pm 7.51\%$, and $19.7\% \pm 7.68\%$, respectively; $P < 0.001$, $P = 0.009$, and $P = 0.011$, respectively). The fructose and atherogenic diet groups also had significant increases in pancreatic fat when compared to the control group ($P = 0.001$ and $P = 0.004$, respectively). Beyond this increase in lipid accumulation, no differences in tissue morphology were observed among the diet groups. Specifically, no inflammation, fibrosis, or cellular injury was observed in any of the test diet groups or controls. Figure 1 shows representative H&E-stained images from all diet groups.

Insulin-positive cells constituted greater than 90% of all islets, and these cells were distributed diffusely throughout the islets. Representative images of insulin staining are shown in Figure 2. Single or small clusters of insulin-immunoreactive cells outside of the islets were also seen scattered throughout tissue samples from all 4 diet groups. In addition, tissue samples from all diet groups contained “microcyst”-like growths within many of the islets (Fig. 2D).

The number of swine scored for each category of islet size and corresponding percentages are shown in Table 3. Five of the 11 control samples scored zero or no enlargement (45%), two scored + (18%), and only one scored +++ (9%; very enlarged). In contrast, 8 of the 9 fructose diet samples showed islet enlargement (89%), and 6 of the 9 atherogenic diet samples were enlarged (67%). Furthermore, all 5 NASH diet samples showed enlargement, and 4 of the samples were scored as +++ (80%).

Oxidative Stress

Oxidative stress, which may play a role in cellular injury, was measured by examining MDA and TEAC levels in serum and pancreatic tissue homogenate (Table 4). The NASH diet group exhibited a significant increase in both the serum and pancreatic tissue levels of MDA ($3.0 \pm 3.3 \mu\text{mol/L}$ and $0.1 \pm 0.12 \text{ nmol/mg protein}$, respectively) compared to the control group ($1.46 \pm 0.32 \mu\text{mol/L}$ and $0.04 \pm 0.01 \text{ nmol/mg protein}$, respectively; $P = 0.006$ and $P = 0.01$, respectively). The ratio of MDA to TEAC was also significantly greater in the NASH diet group (1.3 ± 1.83) when compared to controls (0.41 ± 0.16 , $P = 0.006$). No differences in the serum and pancreatic MDA and the pancreatic TEAC were observed among the fructose, atherogenic, and control diet groups. In addition, the TEAC levels in swine fed with NASH diet ($0.09 \pm 0.01 \mu\text{mol/mg protein}$) were not different when compared to the control, fructose, or atherogenic diet groups (0.1 ± 0.01 , 0.092 ± 0.004 , and $0.12 \pm 0.05 \mu\text{mol/mg protein}$, respectively).

DISCUSSION

In this study, the effect of feeding 3 different obesogenic diets (high-fructose, atherogenic, or NASH diet) on the pancreata of Ossabaw swine was examined. Overall, the fructose diet, which induced metabolic syndrome in the absence of changes in liver histology,⁸ resulted in

enlargement of some islets with lipid accumulation, but no other significant changes within the pancreas, such as cellular injury, inflammation, or lesions, were observed. The atherogenic diet, which induced metabolic syndrome and simple steatosis in the liver,⁸ also induced some islet enlargement and lipid accumulation in the pancreas. The NASH diet, however, induced severe metabolic syndrome and abnormal liver histology consistent with human NASH,⁸ along with islet enlargement and significant fat accumulation in the pancreatic tissue. Interestingly, there was no association between the amount of pancreatic fat and the severity of histological features of NASH, including hepatic fat accumulation, potentially because of our small sample size. The NASH diet group also exhibited significantly increased serum and pancreatic oxidative stress levels in the absence of cellular injury or inflammation in the pancreas.

It has been previously shown that obesity can lead to an increase in pancreatic fat in both animal models and humans,^{3,16-19} and similar observations were made in the current study. However, increased pancreatic fat was not accompanied by cellular injury or inflammation, indicating that simple steatosis alone was not sufficient to induce pancreatitis/NASP in our animal model. In fact, multiple factors are likely influential in the progression of NAFPD to NASP.^{3,16} For example, oxidative stress has been cited as a factor in the pathogenesis and progression of fatty pancreas to pancreatitis.¹⁶ The NASH diet group did exhibit increased oxidative stress; however, progression to pancreatitis had not yet occurred. The serum hormones adiponectin and leptin have also been identified as factors that may contribute to the pathology of pancreatitis⁴ and as markers for pancreatitis,¹⁶ and we observed a significant increase in serum leptin and a borderline significant decrease in adiponectin in swine fed with NASH diet.

The NASH diet group showed the greatest islet enlargement. This trend may depict the pancreas' attempt to compensate for higher glucose levels. Similarly, obese Gottingen miniature swine with metabolic syndrome have also shown β -cell expansion.^{20,21} Whereas female Gottingen miniature swine fed ad libitum for 2 years did not develop type 2 diabetes,²¹ Ossabaw swine have been shown to progress to type 2 diabetes.²² It is therefore possible that dietary intervention for more than 24 weeks, as was done in this study, in Ossabaw swine would stress the pancreas to a sufficient degree to induce pancreatitis.

The pancreata of Ossabaw swine fed with the control diet did contain a small amount of fat and exhibited some islet enlargement, which differs slightly from previous characterizations of porcine pancreatic histology.^{20,23} This could be due to the greater predisposition of Ossabaw swine to develop obesity and metabolic syndrome. Prior studies have identified single or small clusters of β cells outside the islets in porcine pancreases, but it is unknown if they continue to develop into larger or new islets.^{23,24} It is also possible that the small clusters could represent islet degradation or abnormalities, as decreased insulin immunoreactivity in islets was seen in mice fed with a high-fat diet as a sign of islet dysfunction.²⁵ In addition, the origin, cause, and effect of the termed microcystlike growths are uncertain. Cystlike structures have been reported before in porcine islets as a site of new β -cell formation; however, these were larger structures and these observations were made in an in vitro environment.²⁶

In this study, we were able to gain a better understanding of the effects of dietary intervention on the pancreas in our large animal model of obesity, metabolic syndrome, and NASH. In summary, we found that, although the NASH diet did not induce steatopancreatitis, significant fat accumulation and elevated oxidative stress in the pancreas were observed. These important observations suggest that the Ossabaw swine model may better translate to human clinical medicine, as compared to rodent models, because it is only in extreme cases of obesity that lipid accumulates in the pancreas of rodents.⁵ Findings from our study reiterate that the Ossabaw miniature swine is emerging as an important large animal model of obesity, metabolic syndrome,⁵ and NASH,⁸ and extend the characterization of this model to NAFLD, although longer study periods may be required to induce NASH.

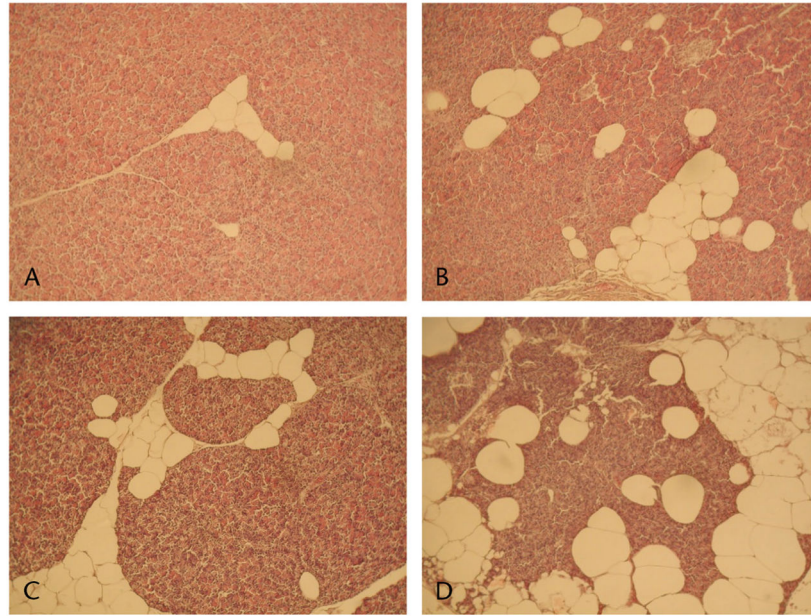
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**FIGURE 1.**

Pancreatic histology. Representative images are shown from the control (A), fructose (B), atherogenic (C), and NASH (D) diet groups. The NASH diet group exhibited significantly greater fat accumulation compared to the control, fructose, and atherogenic diet groups ($P < 0.001$, $P = 0.009$, and $P = 0.011$, respectively). The fructose and atherogenic diet groups had significantly increased pancreatic steatosis compared to the control group ($P = 0.001$ and $P = 0.004$, respectively).

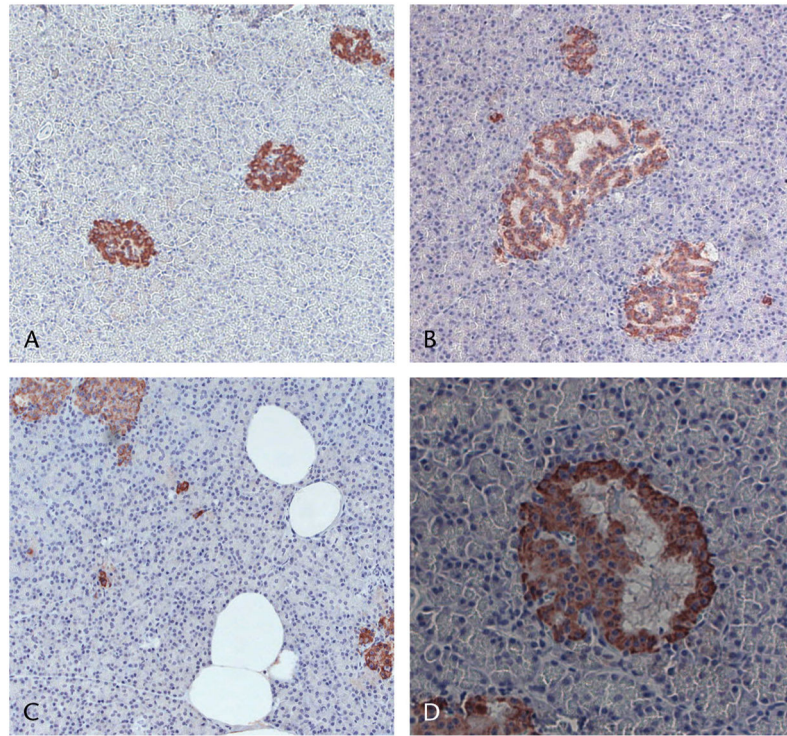


FIGURE 2.

Effects of hypercaloric diets on insulin-positive staining of pancreatic tissue. Representative pancreatic tissue sections stained for insulin (red) with hematoxylin counterstaining (purple) were scored based on the insulin-positive area of islets on a scale ranging from 0 (normal, no enlargement) to +++ (very enlarged): control tissue with no islet enlargement based on insulin-positive area (A), NASH diet group islets with +++ enlargement (B), NASH diet shows fat accumulation within the tissue and several single small clusters of insulin-positive cells (C; A–C, original magnification $\times 200$), and higher magnification view ($\times 400$) of microcystlike growth within islets (D).

TABLE 1

Selected Phenotypic Characteristics of Swine at Sacrifice

	Control Group (n = 12)	Fructose Group (n = 9)	Atherogenic Diet Group (n = 10)	NASH Diet Group (n = 6)
Sex (M/F)	4/8	9/0	4/6	0/6
Weight When Killed, kg	56.9 ± 3	97.7 ± 8.6*	81.1 ± 3.9 [†]	85.6 ± 13.6 ^{*†‡}
Mean Weight Gain, kg	14.2 ± 1.4	52.8 ± 7.3*	35.0 ± 4.3*	37.9 ± 13 ^{*‡}
Serum Glycemic Measures				
Glucose, fasting, mg/dL	77.4 ± 2.7	83.5 ± 2.6	86.4 ± 4.6	87.6 ± 6.4
Insulin, fasting, mg/dL	12 ± 2	15 ± 2	14 ± 1	18 ± 3
HOMA	2.1 ± 0.4	2.8 ± 0.5	3.2 ± 0.3	4.1 ± 1.0*
Peak insulin (by IVGTT), mg/dL	105 ± 9	143 ± 27	113 ± 15	142 ± 25
Plasma Lipids				
Cholesterol, mg/dL	71 ± 4.5	63.0 ± 4.6	401.5 ± 30.9 ^{*†}	628.7 ± 71.9 ^{*†‡}
Triglycerides, mg/dL	24.1 ± 2.5	29.0 ± 2.7	44.7 ± 3 ^{*†}	130.3 ± 16.8 ^{*†‡}
LDL, mg/dL	27.1 ± 3.2	25.3 ± 2.8	280.9 ± 22.9 ^{*†}	519.8 ± 68.3 ^{*†}
HDL, mg/dL	39.2 ± 3.3	31.9 ± 3.0	80.9 ± 5.9	82.8 ± 5.3
Serum Chemistry Profile				
AST, IU/L	35 ± 5	30 ± 6	34 ± 6	100 ± 21 ^{*†‡}
ALT, IU/L	42 ± 6	18 ± 1	30 ± 2	41 ± 12 ^{†‡}
Alkaline phosphatase, IU/L	74 ± 8	60 ± 9	119 ± 9	273 ± 110 ^{*†‡}
Amylase, IU/L	1178 ± 86	1097 ± 137	1070 ± 90	780 ± 110
Lipase, IU/L	25 ± 0.4	25 ± 0	25 ± 0	25 ± 0
Serum Hormones				
Adiponectin (qauc) [§]	16,351 ± 1322	Not done	13,705 ± 894	13,296 ± 663
Leptin, ng/dL	2 ± 0.2	4 ± 1	3 ± 1	17 ± 5 ^{*†‡}

Data are shown as mean ± SD.

* $P < 0.05$ when compared with the control group.

[†] $P < 0.05$ when compared with the fructose group.

[‡] $P < 0.05$ when compared with the atherogenic diet group.

[§] Expressed as protein intensity.

IVGTT indicates intravenous glucose tolerance test; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; AST, aspartate aminotransferase; ALT, alanine aminotransferase.

TABLE 2

Percentage of Fat in Pancreas Tissue

	Percentage of Fat (Area of Fat/Total Area)
Control Group (n = 12)	9.29 ± 1.94
Fructose Group (n = 9)	22.9 ± 7.51*
Atherogenic Diet Group (n = 10)	19.7 ± 7.68*
NASH Diet Group (n = 6)	38.7 ± 15.3* ^{†‡}

Data are shown as mean ± SD.

* $P < 0.05$ when compared with the control group.

[†] $P < 0.05$ when compared with the fructose group.

[‡] $P < 0.05$ when compared with the atherogenic diet group.

TABLE 3

Number of Swine With Islet Cell Size Scores (0 to +++) Based on Insulin-Positive Staining Area

	No Enlargement: 0	Slightly Enlarge: +	Enlarge: ++	Very Enlarged: +++
Control Group (n = 11)	5 (45%)	2 (18%)	3 (27%)	1 (9%)
Fructose Group (n = 9)	1 (11%)	2 (22%)	3 (33%)	3 (33%)
Atherogenic Diet Group (n = 9)	3 (33%)	1 (11%)	2 (22%)	3 (33%)
NASH Diet Group (n = 5)	0	1 (20%)	0	4 (80%)

Data are shown as number of swine (%).

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TABLE 4

Malondialdehyde and TEAC Levels in Serum and Pancreatic Tissue Homogenate

	Control Group (n = 12)	Fructose Group (n = 9)	Atherogenic Diet Group (n = 10)	NASH Diet Group (n = 6)
Serum MDA, $\mu\text{mol/L}$	1.46 \pm 0.32	1.75 \pm 0.54	2.11 \pm 0.48	3.00 \pm 3.3*
Pancreatic MDA, nmol/mg protein	0.04 \pm 0.01	0.048 \pm 0.0125	0.05 \pm 0.01	0.1 \pm 0.12*
Pancreatic TEAC, $\mu\text{mol/mg}$ protein	0.1 \pm 0.01	0.092 \pm 0.004	0.12 \pm 0.05	0.09 \pm 0.01
Pancreatic MDA/TEAC	0.41 \pm 0.16	0.53 \pm 0.15	0.45 \pm 0.13	1.30 \pm 1.83*

Data are shown as mean \pm SD.* $P < 0.05$ when compared with the control group.

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